The role of the vascular K_{ATP} channel in septic models

by

Yi-Ling Chan

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Department of Medicine and
Wolfson Institute for Biomedical Research
University College London

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For Mum, Ulica and Yi-Fan

I, Yi-Ling Chan, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Septic shock is characterized by systemic vasodilatation and an attenuated pressor response to catecholamines. As the hyperactivity (*i.e.* more channels remain in the open state) of the vascular adenosine triphosphate (ATP)-sensitive type of potassium (K_{ATP}) channels results in vasodilatation, this channel has been implicated in the pathogenesis of septic shock. The studies present in this thesis sought to demonstrate that sepsis activates vascular K_{ATP} channels, that channel inhibition reverses hypotension, and that channel hyperactivity is related to an increased gene expression. Rat aortic smooth muscle cells exposed to bacterial endotoxin and the inflammatory mediator interleukin-1β were found to have an increased gene expression of $K_{IR}6.1$, the channel's pore-forming subunit, at 48h. This gene induction could be reversed by the nitric oxide synthase inhibitor, 1400W. $K_{IR}6.1$ mRNA was also increased in mesenteric arteries from rats subjected to faecal peritonitis for 24h. ^{86}Rubidium efflux experiments revealed an increased channel activity in these vessels when stimulated by the K_{ATP}-channel opener, levcromakalim; the basal activity, however, remained unchanged. Conscious rats subjected to faecal peritonitis developed an attenuated pressor response to norepinephrine that, unexpectedly, could not be reversed by the channel inhibitor, PNU-37883A; this agent could not either elevate the baseline blood pressure of septic rats. Surprisingly, this attenuated pressor response to PNU-37883A could be partially reversed by the autonomic ganglion blocker, pentolinium, suggesting that during sepsis the vascular K_{ATP} channel may be inhibited *in vivo* by the high sympathetic tone. Therefore, the inhibition of vascular K_{ATP} channels may assume a limited role in the treatment of septic shock.

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Abbreviations

$[X]_i$	Intracellular concentration of the substance *X*
$[X]_o$	Extracellular concentration of the substance *X*
1400W	*N*-(3(aminomethyl)benzyl)acetamidine
ACE	Angiotensin converting enzyme
ATP	Adenosine triphosphate
BK_{Ca}	Large-conductance, Ca^{2+}-activated K^+ channel
cAMP	Adenosine 3',5'-cyclic monophosphate
CAPS	3-(cyclohexylamino)-1-proprane sulphonic acid
cGMP	Guanosine 3',5'-cyclic monophosphate
CGRP	Calcitonin gene-related peptide
CLP	Caecal ligation and puncture
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
$DiBAC_4(3)$	Bis-(1,3-dibutylbarbituric acid) trimethine oxonol
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
E_K	Equilibrium potential for the K^+ ion
E_m	Membrane potential
FLIPR	Fluorescent imaging plate reader
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
IL-1β	Interleukin-1β
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 1,4,5-triphosphate
K_{2P}	Two-pore domain K^+ channel
K_{ATP}	ATP-sensitive K^+ channel
K_{Ca}	Ca^{2+}-activated K^+ channel
KCO	K_{ATP} channel opener
K_{IR}	Inwardly rectifying K^+ channel
KO	Knockout

K_V	Voltage-gated K^+ channel
L-NAME	N^{ω}-nitro-L-arginine methyl ester
Lev	Levcromakalim
LPS	Lipopolysaccharide
MAP	Mean arterial pressure
MODS	Multiple organ dysfunction syndrome
NDP	Nucleoside diphosphate
NE	Norepinephrine
NO	Nitric oxide
NOS	Nitric oxide synthase
NOx	Nitrate and nitrite
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PKG	cGMP-dependent protein kinase
PLC-β	Phospholipase C-β
PNU-37883A	*N*-(1-Adamantyl)-*N'*-cyclohexyl-4-morpholinecarboxamidine
P_o	Open probability
PVDF	Polyvinylidene difluoride
RASMC	Rat aortic smooth muscle cells
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SEITU	*S*-ethylisothiourea
sGC	Soluble guanylate cyclase
SNP	Sodium nitroprusside
SR	Sarcoplasmic reticulum
SUR	Sulphonylurea receptor
TBS-T	Tris-buffered saline-Tween 20
TEA	Tetraethylammonium
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TNF-α	Tumour necrosis factor-α
VDCC	Voltage-dependent Ca^{2+} channel

Publication

Abstracts

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Chapter One Introduction

Sepsis is the acute systemic inflammation induced by infection that can progress to multiple organ dysfunction (Bone *et al.*, 1992). This dysfunction can include a decrease in vascular tone and the resultant persisting hypotension, "septic shock," a condition that carries a high mortality. As hyperactivity (*i.e.*, more channels remaining in the open state) of the potassium (K^+) channel in the plasma membrane of arterial smooth muscle can cause vasodilatation (Nelson and Quayle, 1995), these channels, particularly the adenosine triphosphate (ATP)-sensitive type (K_{ATP}) of channels, are implicated in the pathogenesis of septic shock (Landry and Oliver, 1992). Since vascular K_{ATP} channel hyper-activity may originate from an increase in channel number or/and channel open probability, the work presented in this thesis sought to understand if sepsis could induce the expression of the channel gene and a subsequent increase in the channel number. I also investigated the feasibility of inhibiting the channel to reverse the vasodilatation and vascular hyporeactivity induced by sepsis.

1.1. Severe sepsis and septic shock: a major medical challenge

Modern medicine uses the term 'sepsis' (Greek: σηψις, the decomposition of organic matter in the presence of bacteria) (Geroulanos and Douka, 2006) to denote the acute systemic inflammation that results from the massive and widespread activation of the immune system by microbial toxins and cell

components (Bone *et al.*, 1992). As a host response aiming to eradicate the pathogen, sepsis is composed of a variety of acute immune reactions involving multiple components and mediators, including monocytes/macrophages, neutrophils, endothelium, cytokines, the complement system, coagulation cascades, lipid mediators, the nervous system, and adaptive immunity (for reviews, see Cohen, 2002; Annane *et al.*, 2005). Responses are both pro-inflammatory to enhance the antimicrobial activity, and concurrently anti-inflammatory to prevent excessive and unfettered inflammation. However, when the pro-inflammatory reaction is excessively strong and uncontrolled by the balancing anti-inflammatory response, sepsis can progress and lead to deleterious consequences.

Sepsis becomes dangerous to the host when acute organ dysfunction develops; this condition is termed 'severe sepsis' (Bone *et al.*, 1992). This organ dysfunction frequently involves the lungs (acute lung injury or, more severely, the acute respiratory distress syndrome), the kidneys (acute renal failure), the coagulation system (disseminated intravascular coagulation), brain and nervous system (encephalopathy, neuropathy), muscle (myopathy) and the liver. Cardio-vascular dysfunction can lead to tissue hypoperfusion and, in some cases, profound hypotension despite adequate fluid resuscitation (septic shock). When more than one organ systems develop dysfunction, the patient is said to have multiple organ dysfunction syndrome (MODS; Bone *et al.*, 1992).

Sepsis can induce numerous pathophysiological abnormalities that can cause organ dysfunction. These include tissue hypoperfusion (Bakker *et al.*, 1996),

derangement of the microcirculation (De Backer *et al.*, 2002), abnormal intravascular coagulation (Bernard *et al.*, 2001), the induction of apoptosis (Hotchkiss *et al.*, 1999), an increase in oxidative stress (Goode *et al.*, 1995), and cellular bioenergetic failure (Brealey *et al.*, 2004). Since this organ dysfunction often renders the body unable to maintain normal homeostasis, a third to half of severely septic patients eventually succumb to death, despite active medical intervention (Levy *et al.*, 2010).

The presence of septic shock is associated with the highest mortality rates, claiming 40-70% of affected victims (Annane *et al.*, 2003). It can probably be considered the major cause of early death from sepsis (Rivers *et al.*, 2001). This poor prognosis persists despite attempts to reverse the shock using fluid resuscitation and potent intravenous (*i.v.*) vasopressor agents such as norepinephrine (NE) and dopamine. The degree to which this is related to the severity of the septic condition itself, or to covert harm from current therapies such as catecholamines (Singer, 2007) is a subject of increasing debate.

1.2. Septic shock is a form of vasodilatory shock

1.2.1. Arterial blood pressure and vascular tone

The main role of the cardiovascular system is to transport oxygen and nutrients to the tissue and to remove waste products. Small arteries and arterioles have small diameters and thus high resistance to blood flow (Badeer, 2001). To overcome this resistance and drive blood flow through these vessels, the arterial pressure is maintained at high levels through the heart's continual

pumping of blood into a tight arterial vasculature, wherein the low compliance generates high pressures from small filling volumes. The arterial vasculature has only a very narrow margin for maintaining adequate pressures volume-wise (Guyton and Hall, 2006). Underfilling of the vasculature as a result of a reduced blood volume (hypovolaemia) or/and a weakened cardiac pump can significantly lower arterial pressure. The volume deficit in question could be no more than a few hundred millilitres.

Arteries are normally maintained in a partially constricted state, termed "vascular tone," which determines the arterial blood pressure at a given cardiac output. In most tissues, the blood vessels (except metarterioles and capillaries) are innervated by sympathetic nerve fibres that transmit continual impulses from the brain stem vasomotor centre to the vessels. These impulses stimulate continual release of NE and other vasoactive substances (*e.g.* ATP) from sympathetic and other nerve endings to induce vascular smooth muscle contraction, through the adrenergic receptor-dependent and independent mechanisms (for review, see Hirst and Edwards, 1989). The frequency of neural impulses is termed "sympathetic tone." More frequent impulses (higher sympathetic tone) stimulate release of more vasoactive substance to cause a greater degree of vascular smooth muscle contraction and vasoconstriction. Conversely, less frequent impulses (lower sympathetic tone) cause smooth muscle relaxation and vasodilatation.

When hypotension develops, the body responds acutely by (i) 'pressurizing' arteries through constricting the vessel and pumping in more blood, and (ii)

increasing water reabsorption from the kidneys through an increase in hormones like vasopressin, angiotensin II and aldosterone. Although arterial constriction may decrease the blood flow through certain parts of the body and thereby cause tissue ischaemia and necrosis, without this constriction the tissue hypoperfusion resulting from hypotension will be even more profound and diffuse (Bonev and Nelson, 1993a). Therefore, rapid and potent haemodynamic homeostasis-maintaining mechanisms sense changes in arterial blood pressure, to which they respond aggressively.

1.2.2. Vascular tone is decreased during septic shock

Several mechanisms act in concert to reverse hypotension and maintain blood pressure (Figure 1.1; for review, see Guyton, 1991). Rapid-acting pressure controllers include the baroreceptor reflex, the chemoreceptor reflex, and the central nervous system ischaemic response. These controllers function through stimulating sympathetic tone, which constricts both the arterial and the venous vasculature and increases myocardial contractility. Hypotension also rapidly stimulates the production of angiotensin II and secretion of vasopressin; both are vasoactive hormones that also constrict arterioles. Arterial vasoconstriction elevates blood pressure, whereas venoconstriction increases cardiac preload. Arterial hypotension can also mobilize blood from a major reservoir of blood volume, the splanchnic veins, into the systemic circulation (Gelman, 2008). The increase in both myocardial contractility and cardiac preload augments cardiac output, which also helps to maintain an adequate arterial pressure. Hypo-volaemia or impaired cardiac pumping usually induces an increased vascular

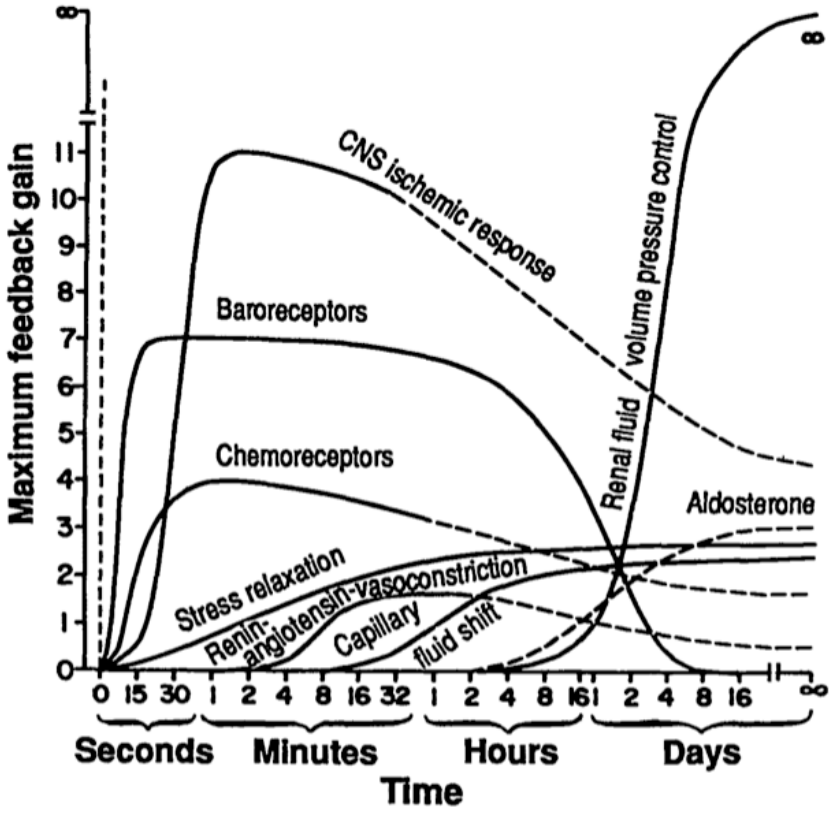


Figure 1.1. Potency of major blood pressure-controlling mechanisms at different time intervals. *Potency is shown in terms of feedback gain, the ratio between changes in blood pressure correctable by the specific mechanism and the final change in pressure that remains. The vertical dashed line indicates a sudden change in blood pressure.* CNS = *central nervous system. Figure reproduced from Guyton (1991) as adapted from Guyton, A. C. (1980):* Arterial Pressure and Hypertension*. Philadelphia, PA, USA: W. B. Saunders and Co. Figure reproduced with permission, copyright 1980, W. B. Saunders and Co.*

tone and vasoconstriction to maintain blood pressure. If these compensatory mechanisms are overwhelmed, blood pressure falls leading to hypovolaemic and cardiogenic shock, respectively. In contrast, septic shock can primarily be characterized by a systemic vasodilatation, *i.e.* the vascular tone is directly decreased. The vascular response to catecholamines (“vascular reactivity”) to therapeutically elevate the arterial pressure (a “pressor” effect) also becomes significantly attenuated. Septic shock is widely regarded as the prototype of

"vasodilatory" shock; other conditions include severe anaphylactoid reactions and spinal cord injury. The relative contributions of sepsis-induced vasodilating stimuli overwhelming blood pressure-maintaining mechanisms versus the degree of dysfunction of the compensatory mechanisms themselves requires further study.

Aside from arterial vasodilatation, sepsis also induces other physiological changes that may contribute to the development of hypotension. These include an increase in vascular permeability, a prominent feature of systemic inflammation that leads to increased capillary leak of fluid and plasma proteins. This results in a decrease in intravascular volume and thus a fall in cardiac preload. Secondly, myocardial depression (Rudiger and Singer, 2007) may become the major haemodynamic abnormality in about 10% of septic shock patients. In addition, changes in the venous system (Jacobsohn *et al.*, 1997), neural reflexes (Banet and Guyton, 1971), and adrenergic receptor number (Carcillo *et al.*, 1988) or function (Sibley and Lefkowitz, 1985) may also contribute to the disease process. Their relative importance and respective effects remain to be clarified.

1.3. How does sepsis affect vascular smooth muscle contractility?

1.3.1. Vascular smooth muscle contraction and relaxation

Vascular smooth muscle contraction is triggered by a rise in intracellular calcium (Ca^{2+}) concentration ($[Ca^{2+}]_i$). Ca^{2+} then binds with calmodulin and the complex activates myosin light chain kinase to phosphorylate the myosin head,

thereby facilitating its repetitive binding to the actin filament; the subsequent attachment-detachment cycling between the two molecules causes smooth muscle contraction. $[Ca^{2+}]_i$ is therefore an important determinant of the contractile status of vascular smooth muscle (Rembold and Murphy, 1988). The resting $[Ca^{2+}]_i$ level in smooth muscle is ~0.1 μM. Smooth muscle contraction and vasoconstriction can be induced by an $[Ca^{2+}]_i$ above ~1 μM, whereas a low $[Ca^{2+}]_i$ ceases to activate the myosin light chain kinase and stops the contracting (shortening) process without relaxing the muscle. Complex mechanisms jointly regulate vascular smooth muscle $[Ca^{2+}]_i$ (for reviews, see Akata, 2007b, 2007a).

The relaxation of vascular smooth muscle depends on the activity of the enzyme, myosin phosphatase, which dephosphorylates the myosin head and thus interrupts its interaction with actins (for review, see Horowitz *et al.*, 1996). An increased activity of myosin phosphatase can counteract that of the myosin light chain kinase, resulting in an attenuated muscle contraction for a given $[Ca^{2+}]_i$. This phenomenon is often described as the 'desensitization' of the contractile apparatus to Ca^{2+}; this serves as another important mechanism to modulate smooth muscle contraction (for review, see Ratz *et al.*, 2005).

Major endogenous vasoconstricting stimuli include an elevated sympathetic tone and circulating hormones such as catecholamines, angiotensin II, and vasopressin. Elevated sympathetic tone not only increases the release of NE from sympathetic nerve endings, but also stimulates the adrenal medullae to secrete NE and epinephrine into the blood. This exerts almost the same effect

as direct sympathetic stimulation (Guyton and Hall, 2006). Angiotensin II and vasopressin are both potent arterial vasoconstrictors, but only angiotensin II can cause moderate venoconstriction (Lee *et al.*, 1988). Importantly, these vasopressors induce vascular smooth muscle contraction principally through a common intracellular signalling pathway: the activation of phospholipase C (PLC)-β by the G_q-protein that couples to their respective receptors in the cell membrane (for review, see Rhee, 2001). PLC-β, through the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate, generates two messengers, *i.e.*, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), the latter going on to activate protein kinase C (PKC). IP_3 elevates vascular smooth muscle $[Ca^{2+}]_i$ principally through inducing the release of Ca^{2+} from the sarcoplasmic reticulum (SR); this accounts for the initial phasic component of contraction. At the same time, a sustained increase in $[Ca^{2+}]_i$ can occur by the influx of extracellular Ca^{2+} down its electrochemical gradient, produced by the action of DAG and PKC on membrane ion channels. This involves activation of both the non-selective Ca^{2+}-permeable cation channel, and the L-type voltage-dependent Ca^{2+} channels (VDCC) by PKC (Nelson *et al.*, 1990; Schöfl *et al.*, 1995; Barrett *et al.*, 2007b). The activity of VDCC can be further enhanced as opening of cation channels will also cause cell membrane depolarization.

There are also important endogenous vasodilators, such as prostacyclin, adenosine and calcitonin gene-related peptide (CGRP). These vasodilators act through binding to their respective receptors that couple to the G_s-protein that subsequently activates the enzyme, adenylate cyclase. This enzyme then synthesizes adenosine 3',5'-cyclic monophosphate (cAMP) from ATP and

subsequently goes on to activate the cAMP-dependent protein kinase (PKA). PKA may phosphorylate myosin light chain kinase and thereby decrease its affinity to Ca^{2+}, leading to a decrease in its activation (Rembold, 1992). PKA also lowers $[Ca^{2+}]_i$ through (i) inhibiting Ca^{2+} entry from the extracellular milieu and Ca^{2+} release from intracellular stores; (ii) facilitating Ca^{2+} clearance from the cytosol; (iii) inhibiting the generation of IP_3 (Murthy *et al.*, 1993); and (iv) activating K^+ channels (Clapp and Tinker, 1998; Ko *et al.*, 2008). These vasodilators are often found to be elevated during sepsis (Bernard *et al.*, 1991; Martin *et al.*, 2000; Muller *et al.*, 2001), but their contribution to the development of sepsis-induced vasodilatation is perhaps not as profound as that of nitric oxide (NO), another potent vasodilator important in the pathogenesis of septic shock (see **1.3.3**).

1.3.2. Voltage dependence of vascular tone

Vascular tone is also dependent on the arterial smooth muscle membrane potential (E_m), and this too may be altered during sepsis. The tonic component of smooth muscle contraction is induced by a tonic increase in $[Ca^{2+}]_i$, which itself is dependent on the activity of L-type VDCC that allows Ca^{2+} entry from the extracellular milieu (Orallo, 1996). The activity of the VDCC is largely dependent on the smooth muscle resting E_m; this is normally about -40 to -60 mV *in vivo*. At the physiological level of vascular smooth muscle E_m there is a steep relationship between it and the corresponding VDCC activity: a change of 3 mV can alter Ca^{2+} influx by as much as twofold (Nelson *et al.*, 1990). Vascular smooth muscle membrane depolarization (*i.e.* a more positive E_m) activates

VDCC and causes smooth muscle contraction, whereas membrane hyperpolarization (*i.e.* a more negative E_m) causes smooth muscle relaxation. This phenomenon is termed “electromechanical coupling” (Somlyo and Somlyo, 1968), and is also an important determinant of vascular tone.

Arterial smooth muscle E_m is determined by the distribution of various kinds of ions across the membrane, and their respective permeability. The principal ions in question are the sodium (Na^+), Ca^{2+}, K^+, and chloride (Cl^-) ions. The membrane conductance associated with K^+ channel activity accounts for about 70% of its total conductance (Nelson and Quayle, 1995), and this is about 10 times that of Cl^- and 200 times that of Na^+ (Hirst and van Helden, 1982). Thus E_m is dominated by the distribution of K^+ across the plasma membrane in smooth muscle, which gives an equilibrium potential (E_K) of -84 mV (Hirst and Edwards, 1989). This predominance of electric conductance for K^+ actually makes the plasmalemmal K^+ channel the major regulator of arterial smooth muscle E_m (Nelson and Quayle, 1995). When more K^+ channels remain in the open state (*i.e.*, hyperactivity), the increased membrane conductance for K^+ drives E_m further toward E_K, and the cell membrane is hyperpolarized. Conversely, when less K^+ channels remain in the open state, E_m then equilibrates toward the equilibrium potential of Cl^- (-31 mV) and the cell membrane is depolarized. Through the subsequent electromechanical coupling, the arterial smooth muscle K^+ channel becomes able to regulate vascular tone. Drugs that open the vascular K^+ channel indeed cause hypotension, whereas drugs that inhibit the channel elevate blood pressure (see below).

Several kinds of K^+ channel are expressed in the arterial smooth muscle cell membrane (for review, see Nelson and Quayle, 1995). These are the voltage-dependent (K_V) channel, the Ca^{2+}-activated (K_{Ca}) channel, the inwardly rectifying (K_{IR}) channel, the K_{ATP} channel, and the two-pore domain (K_{2P}) channel (Gurney and Manoury, 2009). The K_V channel is activated by membrane depolarization, while elevations in $[Ca^{2+}]_i$ activate the K_{Ca} channel. Therefore, these two channels function to prevent excessive depolarization and hence limit smooth muscle contraction. The K_{IR} channel contributes importantly to the resting K^+ conductance, and may be responsible for hyperkalaemia-induced vasodilatation (Quayle *et al.*, 1997). The K_{2P} channel also contributes to background K^+ conductance, and may be involved in arachidonic acid- and polyunsaturated fatty acid-induced arterial dilatation and hypoxia-induced pulmonary vasoconstriction (Gurney and Manoury, 2009). Since many members of this channel family are sensitive to hypoxia and changes in pH, they may also contribute to the development of septic shock but to what extent remains currently unknown. The K_{ATP} channel and, to a lesser extent, the large conductance K_{Ca} (BK_{Ca}) channel have been implicated in the vascular changes and consequent hypotension seen in sepsis (for reviews, see Landry and Oliver, 2001; Buckley *et al.*, 2006), as they can be directly activated by NO, prostacyclin, adenosine and CGRP (Murphy and Brayden, 1995; Nelson *et al.*, 1995; Clapp and Tinker, 1998).

Several studies demonstrated arterial smooth muscle hyperpolarization induced by bacterial endotoxin (lipopolysaccharide, LPS). However, the nature of this hyperpolarization appears heterogeneous, depending on the vessel studied, the

type of septic stimuli, the presence of endothelium in the preparation, and time. (Farias *et al.*, 2002) incubated arterial vessels with LPS for 10 min and found that K_{ATP} channel activity was only involved in hyperpolarization in aortae from spontaneously hypertensive rats (SHR), whereas BK_{Ca} channel activity was involved in mesenteric arterial bed in normotensive rats. NO released from the endothelium did not always stimulate BK_{Ca} channel activity, but was the major mediator of LPS-induced vasorelaxation in SHR. (Chen *et al.*, 2000) found in *ex vivo* aortae from 6h endotoxemic rats that the nitric oxide synthase (NOS; see **1.3.3**) inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME), and the downstream soluble guanylate cyclase (sGC; see **1.3.3**) inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), attenuated LPS-induced hyperpolarization through modulating both BK_{Ca} and K_{ATP} channel activities. (Kuo *et al.*, 2009) found in *ex vivo* aortae from 18h CLP rats that aortic smooth muscle hyperpolarization was attenuated by blockers of BK_{Ca}, K_V and K_{IR} channels, but not every type of K_{ATP} channel blockers. Methylene blue, but not L-NAME or ODQ, could also attenuate the hyperpolarization, These results actually draw more confusion rather than consensus as to the role of vascular K^+ channel in sepsis-induced vascular changes.

1.3.3. Nitric oxide

Probably the most important vasoactive mediator induced by sepsis is NO. This gas has been identified as the endothelium-derived relaxing factor that constitutes a crucial endogenous vasodilator mechanism for regulating blood pressure and flow (for review, see Moncada *et al.*, 1991). NO exerts its

physiological effects principally through activating sGC, which synthesizes guanosine 3',5'-cyclic monophosphate (cGMP) that subsequently activates the cGMP-dependent protein kinase (PKG). In vascular smooth muscle, PKG lowers $[Ca^{2+}]_i$ in many of the same ways as PKA (see **1.3.1**). However, it has the additional capability of inhibiting IP_3-induced Ca^{2+} release through phosphorylating its receptor (Murthy and Zhou, 2003) as well as stimulating Ca^{2+} uptake into the SR by activating the Ca^{2+}/ATPase through phosphorylation of phospholamban, although the latter may also be a target for PKA (Cornwell *et al.*, 1991). The cGMP/PKG and cAMP/PKA signalling pathways can "cross-talk" with each other, and thereby induce similar physiological changes (Lincoln *et al.*, 1990). The cGMP/PKG pathway also modulates myosin phosphatase and PKC activities, and is thereby able to lower Ca^{2+} sensitivity of the muscle (for review, see Carvajal *et al.*, 2000). NO may also react with the superoxide anion to generate the potent oxidizing radical, peroxynitrite, that may cause direct cytotoxicity to vascular smooth muscle leading to contractile failure, cellular energetic failure (Zingarelli *et al.*, 1997) and apoptosis (Li *et al.*, 2004).

NO is synthesized by NOS, of which three isoforms exist. NOS1 (neuronal) and NOS3 (endothelial) isoforms are constitutively expressed, and their activity is dependent on Ca^{2+} and calmodulin. This complex, together with cofactors, stimulates the enzyme to produce a brief production of NO at physiological levels (picomolar to low nanomolar in plasma), where NO functions principally as a neurotransmitter or a signalling molecule. In contrast, NOS2 isoform (otherwise known as iNOS) is not constitutively expressed in most tissues, but is transcriptionally inducible by bacterial endotoxin and certain pro-inflammatory

cytokines. Its activity does not depend on Ca^{2+} (because calmodulin is already tightly bound) and, compared to the other NOS isoforms, can produce hundred-to-thousand-fold higher levels of NO (high nanomolar to low micromolar plasma concentrations), and for many hours. High levels of NO have both antimicrobial and cytotoxic effects. It may also play an important role in shaping the immune response (for reviews, see Fang, 1997; Bogdan, 2001b). However, high levels of NO contribute to the vasodilatation and consequent hypotension seen in sepsis (for review, see Thiemermann, 1997).

Many believe that induction of NOS2 leading to excessive generation of NO represents the primary cause of septic shock. However, in human sepsis, induction of NOS2 appears not to be as widespread or as marked as that seen in rodent models of endotoxic shock (Vallance and Charles, 1998), even though NOS inhibitors are potent pressor agents. In skin/soft tissue biopsies taken from patients with necrotising fasciitis NOS2 activity was found to be compart-mentalized (Annane *et al.*, 2000). In addition, there was a significant inverse correlation between plasma levels of nitrate and nitrite (collectively referred to as NOx, a surrogate marker of *in vivo* NO synthesis) and the mean arterial pressure (MAP) and systemic vascular resistance. However, a clinical trial of a non-specific NOS inhibitor in septic shock patients caused more deaths than the control group treated conventionally with catecholamines (Lopez *et al.*, 2004). In this multi-centre clinical trial, the sepsis-induced vascular changes could not be fully reversed in some patients at the highest dose allowable. This dose should, in theory, be sufficient to block NOS completely. In an animal study using endotoxaemic rats, even when the NOS2 induction was fully

suppressed by pre-treatment with dexamethasone and aminoguanidine, plasma NOx level did not rise yet their aortae still had higher nitrate and cGMP levels, and still showed an attenuated *in vitro* pressor response to NE (Wu *et al.*, 1998). These results could originate from an increase in NOS1 and/or NOS3 activity or some other factor induced by sepsis like carbon monoxide that could stimulate downstream sGC activity (Wu *et al.*, 1994). Notwithstanding this, the plasma NOx level did not appear to reflect these activities.

Considering these results, should more broad-based drugs be used therapeutically, *e.g.* methylene blue (Wu *et al.*, 1998), which will completely suppress both NOS and guanylate cyclase activity? Unfortunately, a complete wipeout of NO may not be totally beneficial, as NO remains important in modulating the immune response. NOS2 gene-knockout (KO) mice can either have a better (Hollenberg *et al.*, 2000), similar (Laubach *et al.*, 1995), or worse (Cobb *et al.*, 1999) outcome after sepsis. Therefore, the potential benefits and risks of modulating NOS and the cGMP/PKG pathway need to be carefully evaluated.

1.3.4. Dysfunction in compensatory mechanisms

As previously described, hypotension may be the result of overproduction of NO (and other mediators) overwhelming the compensatory mechanisms in maintaining blood pressure. Alternatively, it could also be that sepsis renders these mechanisms dysfunctional through affecting the production, receptors or downstream pathways of vasoconstricting hormones. Plasma NE, renin (the

proteolytic enzyme that generates the precursor of angiotensin II) and vasopressin levels were markedly elevated in conscious endotoxaemic rats (Schaller *et al.*, 1985). Receptors for each of these vasoconstrictors are down-regulated during sepsis (Bucher *et al.*, 2001; Bucher *et al.*, 2002; Bucher *et al.*, 2003). Moreover, the activity of the angiotensin converting enzyme (ACE), which catalyses the generation of angiotensin II, was also found to be reduced during experimental and human sepsis (Deitz *et al.*, 1987; Dunn and Horton, 1993). Similarly, plasma vasopressin levels tend to fall back toward baseline after prolonged (≥24h) septic shock in humans (Landry *et al.*, 1997). Thus it may not be surprising that intravenous infusion of angiotensin II (Wan *et al.*, 2009) or vasopressin (Landry *et al.*, 1997) helped to reverse sepsis-induced hypotension. Despite this, a large clinical trial of vasopressin failed to demonstrate an overall survival benefit in septic shock patients compared to NE alone (Russell *et al.*, 2008). However, vasopressin doses used were low and an *a priori* defined subset of patients with lower pressor requirements did show statistically significant survival benefit with vasopressin. Angiotensin II infusion was also shown to beneficially increase urine output and creatinine clearance in experimental hyperdynamic sepsis (Wan *et al.*, 2009). However, one recent animal study showed that administration of ACE inhibitors actually decreased the mortality from multi-organ dysfunction (Schmidt *et al.*, 2010). In summary, derangement of compensatory mechanisms during sepsis may also contribute to arterial vasodilatation, but whether this derangement is primarily induced by sepsis or a secondary change following other insults remains to be clarified.

Experiments performed by Banet and Guyton (1971) elegantly demonstrated the relationships between sympathetic stimulation, cardiac output and arterial blood pressure. Dogs were continuously infused with 2,4-dinitrophenol, a proton ionophore that dissipates the mitochondrial proton motive force and reduces ATP production, thus increasing body oxygen consumption about fourfold. This resulted in diffuse arteriolar dilatation and reflex sympathetic stimulation, a scenario similar to that happens in sepsis. With an intact central nervous system, the dog could increase its cardiac output threefold, and the arterial pressure could be maintained. However, in dogs that had been decapitated and their spinal cords destroyed, the arterial pressure could not be maintained and there was an increase in cardiac output of only 50%. Interestingly, if the decapitated dogs were transfused intra-arterially with blood or blood mixed with dextran solution to maintain their arterial pressure, their cardiac output could also be increased to the same as intact animals. These results suggest that the heart has a functional reserve that can tremendously increase cardiac output to maintain arterial pressure, provided the sympathetic reflex or the intra-arterial blood volume (or the pressure generated from filling the vessels) remains adequate. During septic shock, cardiac output is, in general, not increased as much, so this may also contribute to the development of hypotension. Problems may arise from myocardial depression, suboptimal cardiac preload, derangement of sympathetic reflexes, or/and an increase in arterial compliance. Endotoxin could blunt the muscle sympathetic nerve activity in response to the baroreceptor reflex, and uncouple heart rate modulation with that for blood pressure (Sayk *et al.*, 2008). The baroreceptor reflex may also be affected by angiotensin II (Polson *et al.*, 2007) and vasopressin (Koshimizu *et al.*, 2006).

Therefore, sepsis may render the sympathetic nervous system dysfunctional, and this may also contribute to the development of arterial dilatation and the consequent hypotension.

1.4. Vascular K_{ATP} channels

K_{ATP} channels are so called because they respond to fluctuations in intracellular ATP concentrations ($[ATP]_i$) and open and close in response to metabolic demand (Noma, 1983). Under physiological concentrations (millimolar) of intracellular ATP, channel opening is rare (Quayle *et al.*, 1994). In contrast, nucleoside diphosphates (NDP) are able to activate the channel, even in the presence of normal $[ATP]_i$ (Beech *et al.*, 1993). Thus, the ratio of ATP to NDP within the cell becomes a key determinant of basal activity of the channel in many tissues (Seino and Miki, 2003).

K_{ATP} channels are inhibited by sulphonylurea agents such as glibenclamide, glipizide and tolbutamide which bind to the sulphonylurea receptor (SUR) subunit of the channel (see **1.4.1**). A chemically diverse group of K_{ATP} channel openers (KCOs) that include agents such as levcromakalim, pinacidil, diazoxide, minoxidil and nicorandil also interact with the SUR but in contrast activate the channel and cause substantial hypotension *in vivo* (Mannhold, 2004).

1.4.1. An hetero-octomer of two subunits in a 1:1 stoichiometry

The structure of K_{ATP} channels consists of a hetero-octomer of two subunits in a 1:1 stoichiometry (Figure 1.2). Four subunits belonging to the inwardly rectifying K_{IR}6.x channel protein family form the pore (Inagaki *et al.*, 1995), while 4 SUR regulatory subunits belonging to the ATP-binding cassette family of proteins (Aguilar-Bryan *et al.*, 1995) surround them; both are required to form a fully functional channel (see below). Glibenclamide and KCOs exert their effect through binding to the SUR subunit, as do NDP, whereas ATP binds to the pore-forming subunit to inhibit the channel (Figure 1.2). However, high doses of glibenclamide may also inhibit the pore (Bryan and Aguilar-Bryan, 1999). Isoforms of each of the subunits have been identified: K_{IR}6.1 (encoded by the gene *KCNJ8* in human) and K_{IR}6.2 (*KCNJ11*) for the pore-forming subunit; SUR1 (*ABCC8*) and SUR2 (*ABCC9*) for the regulatory subunit. Two major splice variants exist for the SUR2 gene, giving rise to either SUR2A or SUR2B protein. In different tissues, different compositions of K_{IR}6.x/SUR are represented: K_{IR}6.2/SUR1 channels in pancreatic β-cells and neurons, K_{IR}6.2/SUR2A in cardiac and skeletal muscles and K_{IR}6.1/SUR2B in vascular smooth muscle (for reviews, see Seino and Miki, 2003; Buckley *et al.*, 2006). Another K_{ATP} channel inhibitor particularly important in the current study is PNU-37883A (Humphrey, 1999), which binds specifically to the vascular isoform of the pore-forming subunit (Cui *et al.*, 2003; Teramoto, 2006). Thus it is not surprising that PNU-37883A had little effect on cardiac or β-pancreatic cloned K_{ATP} channels (Cui *et al.*, 2003).

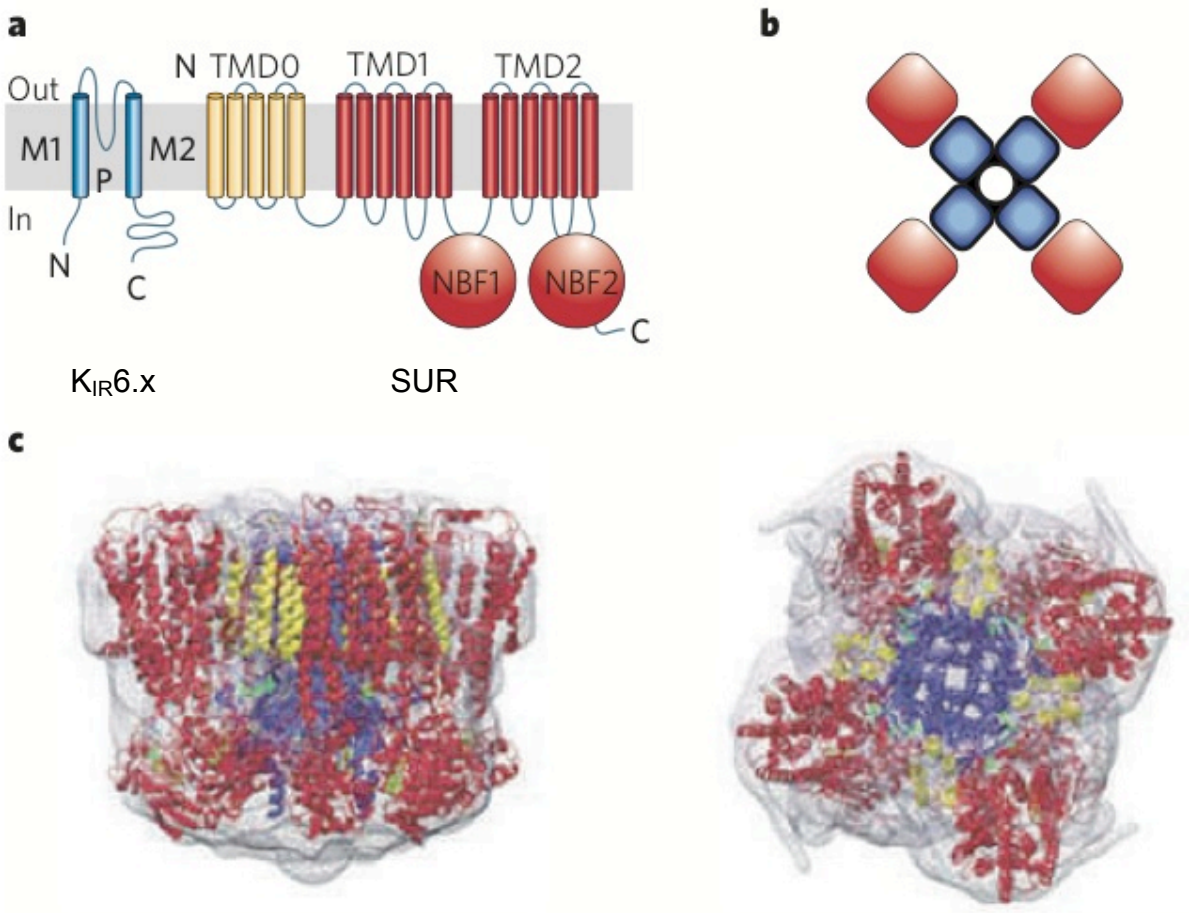


Figure 1.2. Structure of the K_{ATP} channel. a) *K_{ATP} channels are composed of two subunits. The pore-forming subunit consists of K_{IR}6.x, which belongs to the inwardly rectifying K^+channel protein family. The regulatory subunit, termed the sulphonylurea receptor (*SUR*), belongs to the ATP-binding cassette protein family.* TMD = *trans-membrane domain;* NBF = *nucleotide binding fold.* ***b)*** *Functional channels are hetero-octomers, which consist of 4 K_{IR}6.x and 4 SUR subunits in a 1:1 stoichiometry.* ***c)*** *Images at 18Å resolution of the K_{ATP} channel complex in the plane of the membrane (left) and from above the membrane (right). Domains in the 3-dimentional model are represented in the same colour as that for their respective domains in (**a**). Figure reproduced from Nichols (2006) by permission from Macmillan Publishers Ltd.*

Pancreatic K_{ATP} channels dominate the resting K^+ permeability of the β-cell in the absence of glucose (Ashcroft and Rorsman, 1989). Hyperglycaemia leads to an increase in $[ATP]_i$, which inhibits the K_{ATP} channel and thereby depolarize the cell. The resultant activation of the VDCC and increase in $[Ca^{2+}]_i$ stimulate the release of insulin from β-cells (Petit and Loubatières-Mariani, 1992). Myocardial K_{ATP} channels, activated by the raised $[ADP]_i$ resulting from ischaemia, exert protective effects on cardiac muscle through shortening the action potential duration and decreasing Ca^{2+} loading (Nichols and Lederer, 1991). However, certain myocardial KCOs protect ischaemic/reperfused myocardium that appears independent of these two effects, suggesting the existence of other protective mechanisms or sites of action of these agents (Grover and Garlid, 2000). Amongst is the mitochondrial K_{ATP} (mitoK_{ATP}) channel located in the inner membrane of mitochondria, which can be activated by the KCO, diazoxide, at thousand-fold lower concentrations compared to sarcolemmal K_{ATP} channels (Garlid *et al.*, 1996). At this mitoK_{ATP}-selective level, diazoxide exerts protective effects on myocardium during ischaemia, which can be abolished by the inhibitor of the mitoK_{ATP} channel, 5-hydroxydecanoic acid (Garlid *et al.*, 1997). Thus the K_{ATP} channels in different tissues can exert a variety of effects important to normal physiology.

1.4.2. Channel subunit gene expression

According to the AceView annotations of the National Center for Biotechnology Information rat genome database (http://www.ncbi.nih.gov/IEB/Research

/Acembly/ ; Thierry-Mieg and Thierry-Mieg, 2006), the *Kcnj8* gene is expressed at low level, whereas the *Abcc9* is moderately expressed.

Several transcription factors are implicated in regulating K_{ATP} subunit gene expression, for example, the Forkhead (Foxs) (Philip-Couderc *et al.*, 2008); the nuclear factor (NF)-κB (Shi *et al.*, 2010), Sp1 (Ashfield and Ashcroft, 1998; Hernández-Sánchez *et al.*, 1999), E-box and activating protein (AP)-2 (Ashfield and Ashcroft, 1998), Isl-1 (Hashimoto *et al.*, 2005), and hypoxia-inducible factor 1α (Raeis *et al.*, 2010). NF-κB and Foxs are particularly relevant to sepsis, as they can be induced by bacterial endotoxin (Cohen, 2002) or/and the inflammatory mediator, tumour necrosis factor (TNF)-α (Isidoro Tavares *et al.*, 2009). Other factors that have been shown to affect gene expression of these two subunits include hypoxia (Crawford *et al.*, 2003), dexamethasone (Hernández-Sánchez *et al.*, 1999; d'Emmanuele di Villa Bianca *et al.*, 2003), growth hormone (Tivesten *et al.*, 2004), 17β-estradiol (Ranki *et al.*, 2002), high glucose concentrations and diabetes (Moritz *et al.*, 2001; Ren *et al.*, 2003) as well as hypercholesterolaemia (Ren *et al.*, 2001). Importantly, dexamethasone significantly reduced KCO-induced hypotension in endotoxaemic rats in a time-dependent manner, suggesting the presence of *de novo* synthesis of vascular K_{ATP} channels (d'Emmanuele di Villa Bianca *et al.*, 2003).

Importantly, the synthesis of the pore-forming subunit does not necessarily lead to the constitution of more functioning channels. As the $K_{IR}6.2$ subunit rapidly degraded if not binding to SUR1 (Crane and Aguilar-Bryan, 2004), it is likely that the number of the existing SUR molecules actually determines how many

functioning channels can eventually be formed. Indeed, (van Bever *et al.*, 2004) overexpressed $K_{IR}6.2$ subunit by 7-fold, resulting in only a 58% increase in cultured ventricular myocyte K_{ATP} current density. Their result also indicates the presence of a reserve of SUR1 in the myocyte that can accommodate additional $K_{IR}6.2$. Jin *et al.* (2004) saw a 22-fold increase in $K_{IR}6.1$ expression in colonic smooth muscle cells induced by chronic inflammation, concomitant with an increase in K_{ATP} current density by only 2.5-fold. Taken together, the increase in $K_{IR}6.1$ transcriptional expression during sepsis may not correlate well with the concurrent change in K_{ATP} channel activity.

1.4.3. Surface expression and turnover

After gene translation, the subunit proteins are assembled into functional units of $(K_{IR}6.x/SUR)_4$ and trafficked to the plasma membrane to exert their function. $K_{IR}6.x$ and SUR1 subunits both possess an endoplasmic reticulum retention signal that is an arginine-lysine-arginine motif. This is hidden during their correct incorporation into a functional channel, thus granting access to the plasma membrane (Zerangue *et al.*, 1999). For $K_{IR}6.2$/SUR1 K_{ATP} channels, (Crane and Aguilar-Bryan, 2004) showed that after 60 min of labeling with [^{35}S] methionine/ cysteine, the newly synthesized $K_{IR}6.2$ and SUR1 rapidly associated with each other, and their complexes became detectable in 10 min. Then these $K_{IR}6.2$/SUR1 complexes assembled themselves into tetramers, which were then transitted to the Golgi apparatus wherein the SUR was glycosylated. The labeled mature channels appeared within 2h, and reached the maximum

number at about 10h. The estimated half-life for the complete, fully glycosylated channel is about 7.3h.

1.4.4. Physiological roles of vascular K_{ATP} channels

As mentioned previously, K_{ATP} channels are inhibited by intracellular ATP and sulphonylurea agents, and opened by NDP and KCOs. However, K_{ATP} channels do not simply respond to cell metabolism, since other factors including kinases (Standen and Quayle, 1998; Thorneloe *et al.*, 2002; Lin *et al.*, 2004), phosphatases (Wilson *et al.*, 2000), G-proteins (Terzic *et al.*, 1994), and phospholipids (Baukrowitz and Fakler, 2000) also regulate channel activity and contribute to physiological regulation (Figure 1.3). Likewise, during conditions of metabolic stress such as ischaemia or hypoxia, where $[ATP]_i$ falls and $[NDP]_i$ rises, channel opening becomes substantial (Rodrigo and Standen, 2005). This serves to match blood flow to metabolic demand.

In arterial smooth muscle, K_{ATP} channels contribute to the maintenance of resting E_m and local blood flow. This is based upon observing significant effects of glibenclamide on E_m, vascular resistance, and blood vessel diameter (Bonev and Nelson, 1993a; Jackson, 1993; Duncker *et al.*, 2001). K_{ATP} channels can be activated by vasodilator hormones elevating cAMP (*e.g.*, CGRP, adenosine, and prostacyclin) or cGMP (*e.g.*, atrial natriuretic peptide and NO) (Kleppisch and Nelson, 1995; Quayle *et al.*, 1997). By contrast, vasoconstrictor agents activating PKC (*e.g.* endothelin, angiotensin II, vasopressin) close these channels (Nelson and Quayle, 1995) (Figure 1.3). Thus, the K_{ATP} channel not

only regulates E_m in normal physiology, but also serves as a target for various circulatory stimuli and pharmacological tools.

The physiological role of vascular K_{ATP} channels has been further elucidated using a gene-knockout approach. Both $K_{IR}6.1$ and SUR2 KO mice were prone to sudden death, and this was attributed to coronary vasospasm owing to the disruption of the channel (Chutkow *et al.*, 2002; Miki *et al.*, 2002). The coronary vasospastic episodes were reduced by nifedipine, a L-type VDCC blocker, confirming the link between VDCC and K_{ATP} channel activity. SUR2 KO and $K_{IR}6.1$ KO mice also developed hypertension (Chutkow *et al.*, 2002; Kane *et al.*, 2006). In addition, enhanced expression of $K_{IR}6.1$ and SUR2B subunits induced by growth hormone significantly correlated with a reduction in blood pressure (Tivesten *et al.*, 2004). Thus, vascular K_{ATP} channels seem to play an active role in modulating blood pressure in normal physiology.

Of note, the activity of vascular K_{ATP} channels can be inhibited by an elevated $[Ca^{2+}]_i$. The host lab previously found that the K_{ATP} current of RASMC was almost completely inhibited by a raised $[Ca^{2+}]_i$ (IC_{50} = 100 nM). This inhibition appears to relate to the Ca^{2+}-dependent protein phosphatase-2B, calcineurin, as cyclosporin A, FK-506 or calcineurin autoinhibitory peptide that inhibits calcineurin could increase K_{ATP} channel activity (Wilson *et al.*, 2000). Therefore, vasoconstricting stimuli can inhibit the channel not only through increasing PKC activity but also through increasing $[Ca^{2+}]_i$.

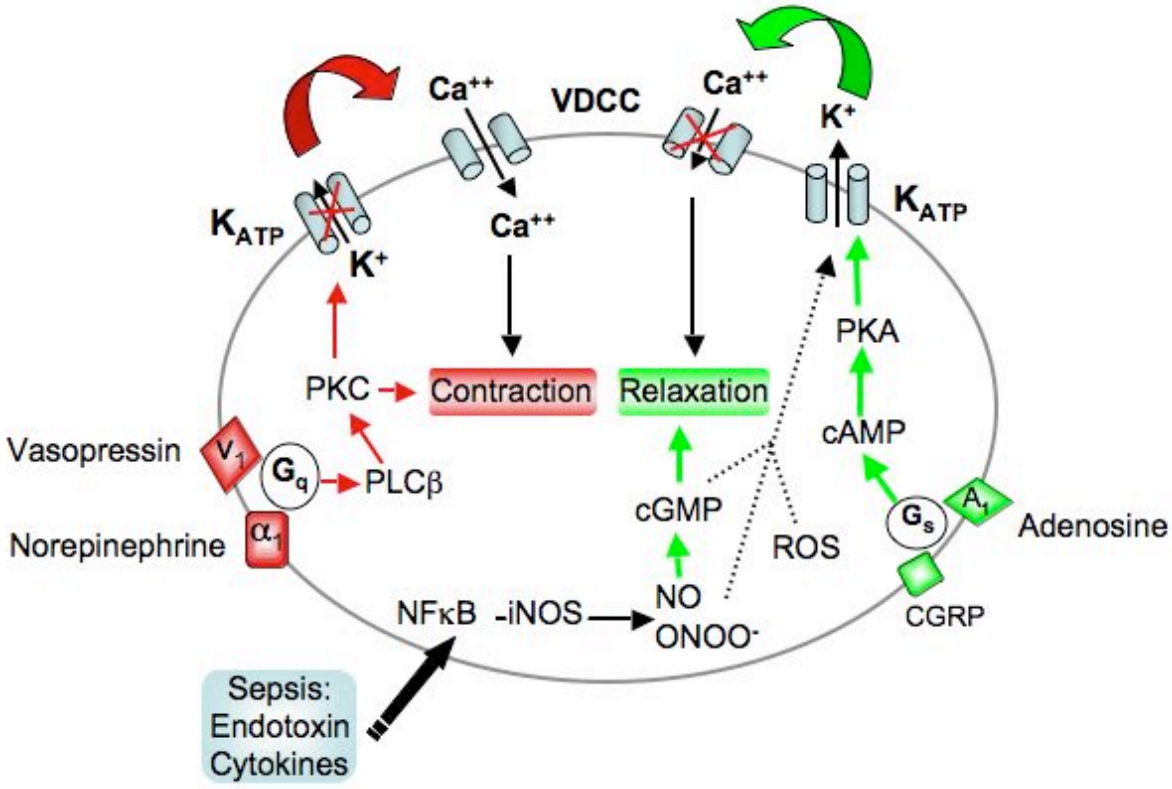


Figure 1.3. Modulating pathways of K_{ATP} channels in arterial smooth muscle. *Vasoconstrictors, through phospholipase C-β (*PLCβ*), activate protein kinase C (*PKC*), which phosphorylates the K_{ATP} channel, closing it and thereby causing depolarization and the opening of the voltage-dependent Ca^{2+} channel (*VDCC*). Vasodilatory receptors activate the K_{ATP} channel through adenosine 3',5'-cyclic monophosphate (*cAMP*)-dependent protein kinase (*PKA*)-dependent phosphorylation. Nitric oxide (*NO*), peroxynitrite (*$ONOO^-$*), reactive oxygen species (*ROS*) and guanosine 3',5'-cyclic monophosphate (*cGMP*) combine to activate the K_{ATP} channel in sepsis. (Adapted from Buckley* et al.*, 2006, by permission of the European Society of Cardiology).*

1.5. Vascular K_{ATP} channel hyperactivity during septic shock

Increasing evidence suggests that abnormal activation of K_{ATP} channels contributes to sepsis-induced cardiovascular collapse (for review, see Buckley *et al.*, 2006). Sepsis induces release of many mediators known to activate K_{ATP} channels (*e.g.*, CGRP, adenosine), whereas other factors, such as acidosis, hyperlactataemia, changes in intracellular adenosine nucleotide composition, and excessive NO generation may provoke further K_{ATP} channel hyperactivity.

However, this notion is not without challenge. Many, but not all, *in vivo* animal models of LPS-induced shock have shown that hypotension could be restored by administration of the K_{ATP} channel inhibitor, glibenclamide, at dosages of 10-40 $mg \cdot kg^{-1}$ *i.v.* (Landry and Oliver, 1992; Gardiner *et al.*, 1999; Sorrentino *et al.*, 1999) Glibenclamide also increased pressor responses to α_1-adrenoreceptor agonists (Landry and Oliver, 1992; Sorrentino *et al.*, 1999). This is in direct contrast to all *in vitro* and *ex vivo* tissue models to date, where glibenclamide failed to reverse vascular hyporeactivity (*e.g.*, Taguchi *et al.*, 1996; Sorrentino *et al.*, 1999). Similarly, it does not show any pressor effect in patients with septic shock (Warrillow *et al.*, 2006; Morelli *et al.*, 2007; Singer *et al.*, 2005), although it markedly reversed vascular hyporeactivity and reduced catecholamine requirements in hypo-tensive patients with excessive pharmacologic K^+ channel activation (Singer *et al.*, 2005). To address this discrepancy, the host lab used an *in vitro* organ culture model of rat mesentery artery and found that channel inhibitors acting on the pore-forming subunits (namely, PNU-37883A and the barium ion) could fully reverse LPS-induced vascular hyporeactivity at 6h, and

remained able to partially reverse hyporeactivity at 20h (O'Brien *et al.*, 2005). Similar findings were observed in rat thoracic aortae (Wilson and Clapp, 2002), where only K_{ATP} channel antagonists acting on the pore could reverse the NOS2-mediated relaxation. In the latter study, the ability of glibenclamide to reverse KCO relaxation was greatly diminished in the presence of LPS whereas the pore blockers remained relatively effective. Moreover, mice lacking the $K_{IR}6.1$ gene did not become hypotensive following endotoxin administration ((Kane *et al.*, 2006). These results not only suggest the involvement of the K_{ATP} channel in the pathogenesis of sepsis-induced vascular hyporeactivity, but also indicate that K_{ATP} hyperactivity might originate from either a defect in the regulatory function of the SUR subunit, a defect in the communication between the pore and the regulatory subunits, and/or mechanisms that involve the pore-forming subunit only.

The host lab continued to investigate the *in vivo* pressor effect of PNU-37883A and high-dose glibenclamide in a rat model of endotoxic shock (O'Brien *et al.*, 2009). Rats were anaesthetized with isoflurane and fluid resuscitated after *i.v.* infusion of *Klebsiella pneumoniae* endotoxin. Drugs were infused two hours later. While PNU-37883A and high-dose glibenclamide were capable of increasing arterial pressure, a more profound pressor effect was observed in sham-operated rats. This goes against the hypothesis that sepsis can induce vascular K_{ATP} channel hyperactivity, in which case an accentuated pressor response should be present in endotoxic rats. Aside from the theoretical plausibility that the vascular K_{ATP} channel can be activated by sepsis and cause vascular changes pathognomonic to septic shock, it seems never known

whether channel hyperactivity is actually present and to which extent does this contribute to the vascular changes during sepsis.

One concern has been the anesthetics used in this study, isoflurane, which is a K_{ATP} channel activator (Iida *et al.*, 1998) and may thus potentiate the sham rat's pressor response to K_{ATP} channel inhibition. Except for two studies (Gardiner *et al.*, 1999; Lange *et al.*, 2006), all studies showing an enhanced pressor effect of glibenclamide in septic animals were performed in anaesthetized animals. Importantly, most if not all anaesthetic agents appear capable of modulating vascular K_{ATP} channel activity (Kato *et al.*, 2000; Kawano *et al.*, 2004, 2005; Nakamura *et al.*, 2007). Anaesthesia may also affect the reflex control of blood pressure, posing additional confounding effects on the experimental results (Matsukawa and Ninomiya, 1989).

Supposing K_{ATP} channel hyperactivity is induced during sepsis, what might be its origin? Upregulation of channel function may relate to increased channel opening and/or channel number. Enhanced KCO relaxation, vascular hyporeactivity and the pressor effect of glibenclamide at 24h after LPS could be prevented by pre-treatment with dexamethasone (d'Emmanuele di Villa Bianca *et al.*, 2003), suggesting *de novo* synthesis of channel subunits during sepsis. Indeed, messenger RNA (mRNA) levels for $K_{IR}6.1$ increased in the diaphragm of LPS-treated rats at 24h (Czaika *et al.*, 2000), in mice with experimental colitis (Jin *et al.*, 2004), and following ischaemia/reperfusion in rat heart (Akao *et al.*, 1997). Therefore, one possible cause of K_{ATP} channel hyperactivity during sepsis might be an enhanced transcriptional expression of K_{ATP} subunit genes.

Shi *et al.* (2010) showed that murine aortae exposed *in vitro* to LPS (1 $\mu g \cdot ml^{-1}$) for 20 h had a one- to twofold increase in $K_{IR}6.1$ gene expression, and a 50% increase in SUR2B expression. This channel induction was through NF-κB activity, and was accompanied by a 50% increase in basal K_{ATP} current density. I sought to confirm the presence of a similar channel subunit gene induction *in vivo*.

1.6. Summary and aims

Septic shock is characterized by systemic vasodilatation, which may originate from the excessive production of NO, derangement of compensatory mechanisms, and activation of the vascular K_{ATP} channel. Current data remain controversial as to the extent to which K_{ATP} channel hyperactivity contributes to the development of hypotension, and the feasibility of inhibiting the channel to restore blood pressure and the pressor response to catecholamine. *In vitro* experiments demonstrated the presence of vascular K_{ATP} channel hyperactivity induced by LPS that is related to an enhanced channel subunit gene expression. Does the same phenomenon occur in septic patients or animals *in vivo*?

The aims of this thesis are to investigate whether sepsis increases vascular K_{ATP} channel number through inducing channel gene expression, and the feasibility of inhibiting the channel to reverse the sepsis-induced vascular changes. The first aim was to demonstrate the presence of vascular K_{ATP} channel hyperactivity and a corresponding increase in gene expression, using

an *in vitro* primary cell culture model and an *ex vivo* model of arteries taken from septic rats. The second aim of this project was to explore the feasibility of *in vivo* vascular K_{ATP} channel inhibition as a treatment modality to septic shock, especially through using PNU-37883A to block the pore. I observed the respective effect of PNU-37883A and other study drugs on arterial blood pressure and the pressor response to catecholamines in a fluid-resuscitated rat model of peritonitis. In light of the potential confounding effects from the anaesthetic agents used in previous studies, I chose to observe these events in conscious rats.

Chapter Two Methods and Experimental Protocol

I initially used a primary cell culture model of rat aortic smooth muscle cells (RASMC) to observe the effect of inflammatory stimuli on vascular K_{ATP} channel expression and functional activity. After finding increased channel gene expression using reverse transcription polymerase chain reaction (RT-PCR) and a corresponding increase in channel activity, I subsequently observed the level of channel gene expression and activity in arterial smooth muscle taken from septic animals. For this purpose I set up an animal model that mimics clinical sepsis as the source of *ex vivo* arterial tissues. Finally, I sought to establish the therapeutic efficacy of the K_{ATP} channel pore blocker, PNU-37883A, in reversing sepsis-induced hypotension and vascular hyporeactivity in this *in vivo* model.

Figure 2.1 schematically shows the protocol I used in the *in vivo* study that utilized a 24h conscious rat model of faecal peritonitis. I observed the ability of NOS inhibition or vascular K_{ATP} channel blockade to reverse the sepsis-induced vascular changes, namely vasodilatation and consequent hypotension, and the attenuated pressor response (*i.e.* vascular hyporeactivity) to catecholamines. I then sacrificed the animals and collected their thoracic aortae and mesenteric arteries to measure levels of vascular K_{ATP} channel gene expression and functional activity. For this latter study, no previous drug treatment was given to the animals prior to tissue collection.

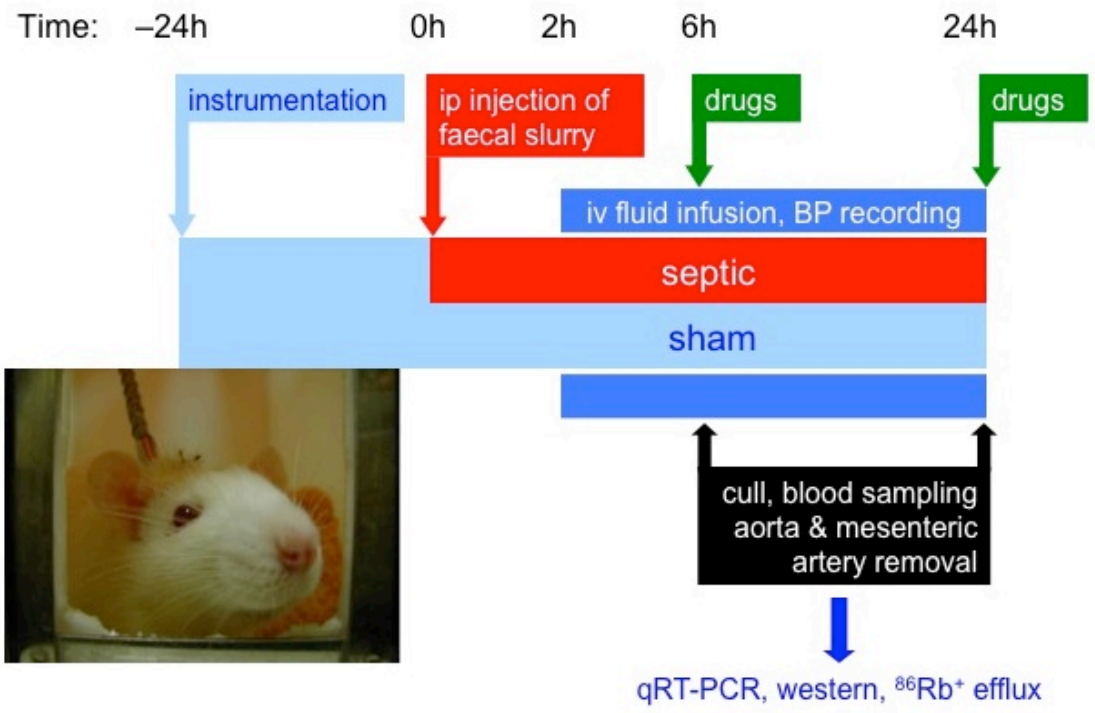


Figure 2.1. Schematic of the protocol used for in vivo experiments. ip = *intraperitoneal;* iv = *intravenous;* BP = *blood pressure;* qRT-PCR = *quantitative reverse transcription polymerase chain reaction;* $^{86}Rb^{+}$ = ^{86}*rubidium.*

2.1. Primary culture of rat aortic smooth muscle cells

2.1.1. Cell harvest and characterization

RASMC were isolated using a method devised by (Clapp and Gurney, 1991). Briefly, male Wistar rats (Charles River, Margate, Kent) of 200-250g body weight were killed by cervical dislocation after stunning. The aorta was excised and kept in ice-cold normal Ca^{2+} solution (in mM: NaCl 112, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1, $NaHCO_3$ 15, NaH_2PO_4 0.5, KH_2PO_4 0.5, glucose 11, 4-(2-hydroxy-ethyl)-1-piperazineethane-sulphonic acid [HEPES] 5, phenol red 0.04; pH 7.3). After dissecting off the surrounding tissue, the aorta was opened longitudinally,

and the endothelium removed by rubbing with a cotton swab. The media layer was carefully stripped off the adventitial layer and separated into ~3 × 3-5 mm pieces. These were washed twice in the dissociation medium (in mM: NaCl 110, KCl 5, $CaCl_2$ 0.16, $MgCl_2$ 2, $NaHCO_3$ 10, NaH_2PO_4 0.5, KH_2PO_4 0.5, glucose 10, HEPES 10, ethylenediaminetetraacetic acid [EDTA] 0.49, taurine 10, phenol red 0.04; pH 7.0 after CO_2 bubbling for 30 min just before use) and then incubated overnight (for about 17h) at 4ºC in 5 ml of fresh dissociation medium containing 0.4 mg·ml^{-1} papain (derived from *Carica papaya*, activity 11.5 U·mg^{-1}; Fluka, Gillingham, Dorset) and 0.02% bovine serum albumin (BSA; essentially fatty acid free). The next morning, 0.1 mM dithiothreitol (DTT) was added to the dissociation medium, and the muscle pieces incubated at 37ºC in a shaking water bath at 40-50 oscillations per min for 15 min. After incubation the muscle pieces were transferred to fresh dissociation medium and gently triturated with a wide-bore (2-3 mm) Pasteur pipette. Relaxed single cells were thus isolated and then plated in a T25 flask in the dissociation medium. After cells had been allowed to settle, the medium was changed to Smooth Muscle Basal Medium (Lonza, Slough, Berks) supplemented with Smooth Muscle Growth Medium-2 SingleQuots (Lonza) that contains human epidermal growth factor, insulin, human fibroblast growth factor-basic, fetal bovine serum (FBS) and gentamicin/ amphotericin-B. Three days later, the medium was switched to Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (PAA Laboratories, Yeovil, Somerset) supplemented with 10% FBS (Gibco, Paisley, Renfrew), 50 units·ml^{-1} penicillin, and 50 µg·ml^{-1} streptomycin (Gibco). Ninety seven percent of cells were characterized as smooth muscle cells by morphology and immunostaining with antibodies (Sigma-Aldrich, Gillingham, Dorset) to smooth muscle α-actin.

2.1.2. Cell culture

RASMC were plated out on Day 0 and were grown in DMEM/Ham's F-12 supplemented with 10% FBS (PAA Labs), 50 units·ml^{-1} penicillin, and 50 μg·ml^{-1} streptomycin. Culture medium was changed on Days 3 and 5, and the cells were then subcultured on Day 7. Cells were washed twice with pre-warmed phosphate buffered saline (PBS; Gibco), and were then trypsinized with 0.05% trypsin/0.53 mM EDTA (Gibco) (2 ml per T-75 flasks) for less than 5 min. Five volumes of DMEM/Ham's F-12 containing 10% FBS was then added to neutralize the activity of trypsin, and the cells were then pelleted by centrifugation at 200*g* for 3 min. The cells were then resuspended in DMEM/Ham's F-12 containing 10% FBS, 50 units·ml^{-1} penicillin and 50 μg·ml^{-1} streptomycin and subcultured at a split ratio of 1:5. For cells subject to experiments, low-endotoxin FBS (PAA labs) was used for subculture.

RASMC between passages 5 and 12 (inclusive) were used for all experiments. When reaching ~80% confluence (on Days 7–8), cells were serum-starved by growing in DMEM/Ham's F-12 containing 0.4% low endotoxin FBS, 50 units·ml^{-1} penicillin and 50 μg·ml^{-1} streptomycin for 48h before giving the septic stimulus (see below). The purpose of serum starvation was to render RASMC quiescent and to synchronize the growth status among cells to the G_0 phase. I wished to exclude the possible effects of growth on K_{ATP} subunit gene expression as the host lab had previously reported that this down-regulated K_{ATP} channel activity (Cui *et al.*, 2002). Indeed, I observed the current set of experiments that growing cells contained only a weak message for $K_{IR}6.1$ (data not shown).

2.1.3. Cell preparation for experiments

For each sample in each condition, a T-75 flask of RASMC grown to 70-80% confluence was used. Quiescent RASMC were treated with a combination of *Salmonella typhosa* LPS (1 $\mu g \cdot ml^{-1}$; Sigma-Aldrich) and interleukin (IL)-1β (10 $ng \cdot ml^{-1}$; R&D Systems, Abingdon, Oxon) in DMEM/Ham's F-12 containing 0.4% low-endotoxin FBS for 48h. A batch of RASMC was pre-treated with the selective NOS2 inhibitor *N*-(3(aminomethyl)benzyl)acetamidine (1400W, 10 μM; Sigma-Aldrich) for 1h before and during the entire period of LPS and IL-1β treatment.

The effect of the septic stimulus on cell viability was assessed with the trypan blue dye-exclusion method (Freshney, 2005). Briefly, RASMC were washed with PBS, trypsinized, and then resuspended in serum-containing culture medium as above. Pelleted cells were resuspended by mixing an equal volume of cell culture medium and 0.5% trypan blue dye to constitute a cell suspension at a density of 5×10^5 $cells \cdot ml^{-1}$. Cells were incubated for 2 min at room temperature, and then 10 μl of the cell suspension transferred to the haemocytometer (Improved Neubauer). Viable cells are impermeable to trypan blue, whereas damaged cells become stained as they take up the dye due to a breakdown in membrane integrity. The total number of cells and the number of stained cells were counted and the mean value of 4-8 counts taken.

2.2. *In vivo* rat model of faecal peritonitis

2.2.1. Animal preparation

Experiments were performed with Home Office (United Kingdom) approval (Licence Number: PPL 70/6143; Principal Licence Holder: M Singer) in accordance with the Animal (Scientific Procedures) Act 1986. Male Wistar rats (Charles River) of ~300g body weight were used throughout. Before instrumentation, animals were housed in the local animal unit for five days' prior, where they kept on a 12:12-h light-dark cycle with free access to food and water.

Under 2% isoflurane (Abbott Laboratories, Queensborough, Kent) anaesthesia maintained via a face mask, the rats were instrumented as follows: the internal jugular vein was cannulated for fluid and drug administration, and the carotid artery cannulated for blood pressure measurement and blood sampling. The catheters (internal diameter, 0.58 mm, external diameter, 0.96 mm) were tunneled subcutaneously to emerge at the nape of the neck where they were mounted onto a swivel/tether system that enabled the rat, on recovery from anaesthesia, to have unimpeded movement around the cage and free access to food and water. Both catheters were flushed continuously with 0.15 $ml \cdot h^{-1}$ heparinized saline (1:1,000). Mean arterial pressure (MAP) was measured (P23XL transducers, Viggo-Spectramed, Oxnard, CA, USA) and recorded onto a pre-calibrated PowerLab system (ADInstruments, Sydney, Australia). Heart rates were derived from the pressure trace records in some of the animals.

After instrumentation the rats were allowed to wake from anaesthesia and stabilize for 24h, then peritonitis was induced by intraperitoneal injection of faecal slurry (4g per kg body weight) prepared from the bowel contents of a rat from the same batch. The faecal material was suspended in 0.9% saline to constitute 66.7% (w/v) slurry, and then filtered to remove fibrous material. Sham-operated control animals received no intraperitoneal injection to avoid accidental bowel perforation, but were otherwise treated identically. Fluid resuscitation (1:1 solution of 6% hydroxyethyl starch [Voluven, Fresenius Kabi, Warrington, Cheshire] and 5% glucose) was given 2h later at 10 $ml \cdot kg^{-1} \cdot h^{-1}$ via the internal jugular venous catheter.

2.2.2. *In vivo* drug administration

The baseline MAP and the changes in MAP induced by various drugs administered intravenously were assessed at either 6 or 24h after faecal slurry injection. This was performed after arterial and venous catheter patency was confirmed and a stable baseline MAP recorded. Drugs for *in vivo* use were diluted in 0.9% saline unless otherwise specified, and were administered through a 5-min intravenous (*i.v.*) infusion with the regular infusion rate of 10 $ml \cdot kg^{-1} \cdot h^{-1}$ was maintained, *i.e.* no additional volume was administered. Each drug infusion was followed by continuous fluid administration to ensure all active compound had travelled through the catheter system. The plateau of the resultant blood pressure response was recorded.

The following drugs were administered, and their effects on baseline MAP and vascular reactivity to vasoconstrictors assessed:

(1) Norepinephrine (NE; 0.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ *i.v.*): diluted in 5% glucose.

(2) *S*-ethylisothiourea (SEITU; 0.1 $mg \cdot kg^{-1} \cdot min^{-1}$ *i.v.*): a potent, non-selective NOS inhibitor (Boer *et al.*, 2000).

(3) PNU-37883A (1.5 $mg \cdot kg^{-1}$ and 1.0 $mg \cdot kg^{-1} \cdot h^{-1}$ *i.v.*; O'Brien *et al.*, 2009): the vascular selective K_{ATP} channel inhibitor that acts on the pore-forming $K_{IR}6.1$ subunit (Humphrey, 1999; Wellman *et al.*, 1999; Cui *et al.*, 2003).

(4) Levcromakalim (Lev; 150 $\mu g \cdot kg^{-1}$ *i.v.*): a specific K_{ATP} channel opener (Hamilton *et al.*, 1993). Dissolved in 50% (v/v) polyethylene glycol 200/ 0.9% saline as 3 $mg \cdot ml^{-1}$ stock solution, which was further diluted with 0.9% saline to constitute the working solution.

(5) Midazolam (0.2 $mg \cdot kg^{-1}$ and 0.2 $mg \cdot kg^{-1} \cdot h^{-1}$ *i.v.*): a benzodiazepine that exerts a sedative but no analgesic effect. Midazolam has been shown to not alter vascular K_{ATP} channel activity *in vitro* (Nakamura *et al.*, 2007).

(6) Pentolinium (2 $mg \cdot kg^{-1}$ and 2 $mg \cdot kg^{-1} \cdot h^{-1}$ *i.v.*): an autonomic ganglionic blocking agent that binds to the nicotinic acetylcholine receptor, thereby inhibiting the release of epinephrine and NE from adrenergic nerves. Although pentolinium also blocks parasympathetic tone, this effect is often far overshadowed by its effect of sympathetic blockade (Waldman and Pelner, 1956; Guyton and Hall, 2006);

(7) Sodium nitroprusside (SNP; 50 $\mu g \cdot kg^{-1} \cdot min^{-1}$ *i.v.*): a nitrovasodilator;

(8) Hydralazine (300 $\mu g \cdot kg^{-1} \cdot min^{-1}$ *i.v.*): a vasodilator that directly relaxes arterial smooth muscle.

Dose-response curves were created for PNU-37883A (0.001-10 mg·kg^{-1} *i.v.*) in sham and septic rats at 6h (*n* = 4), and Lev (0.01-300 µg·kg^{-1} *i.v.*) in sham and septic rats at 6 and 24h (*n* = 3). The dose of PNU-37883A and Lev that provoked a change in MAP halfway between baseline and maximum changes (EC_{50}) were calculated using the five-parameter logistic equation (Equation 2.1; Giraldo *et al.*, 2002; Prism, GraphPad Software, La Jolla, CA, USA).

A separate batch of rats was pre-treated with reserpine, 4.5 mg·kg^{-1} subcutaneously 48h & 24h prior to and during instrumentation (Martínez-Olivares *et al.*, 2006) to deplete their NE reserves from the sympathetic nerves. Their responses to PNU-37883A and SEITU at 6h after peritonitis were observed.

$$y = \mathrm{MAP}_{\mathrm{Baseline}} + \frac{\mathrm{MAP}_{\mathrm{Max}} - \mathrm{MAP}_{\mathrm{Baseline}}}{\left[1+10^{(\log X_b - x) \times \text{Hill slope}}\right]^S}$$

$$\log X_b = \log EC_{50} + \frac{\log(2^{\frac{1}{S}} - 1)}{\text{Hill slope}}$$

Equation 2.1. Five-parameter logistic equation. *X_b indicates the inflection point of the curve; S is the unitless symmetric parameter, with a value of 1 indicating a symmetric curve (Giraldo* et al.*, 2002).*

2.2.3. Blood and tissue sampling

In vivo arterial blood sampling was performed at predetermined time points when line patency allowed. Small volumes (~0.2 ml) were collected in heparinized capillary tubes for arterial blood gas analysis (ABL-70 analyzer, Radiometer, Copenhagen, Denmark), which included measurement of arterial base excess, lactate, and PO_2. For plasma NOx measurement, blood was obtained by cardiac puncture under anaesthesia at the time of sacrifice. Samples were drawn into heparin sodium (CP Pharmaceuticals, Wrexham, Wrexham County), 40 $U \cdot ml^{-1}$ blood, and were centrifuged at 1,600*g* for 10 min. The plasma supernatant was then collected and frozen at -80ºC.

For quantitative RT-PCR experiments, mesentery extending from the Treitz ligament to the terminal ileum was excised off the bowel along its connection. Then the whole piece of mesentery with the enclosed main trunks and branches of the mesenteric artery and vein was excised and submersed immediately into excessive volume (~5 ml) of RNA*later* RNA-stabilization solution (Ambion, Warrington, Cheshire), which works by preserving the quality and quantity of RNA through inactivation of RNase, the enzyme that degrades RNA. The thoracic aorta was freed from the surrounding tissue and inferior vena cava while *in situ*, and then excised and submersed immediately into RNA*later* reagent. The tissue samples were kept at 4ºC overnight. The next day, the main trunk and branches of mesenteric artery were isolated by dissecting away the surrounding tissue and veins in ice-cold RNA*later* reagent. The media layer of the aorta was stripped off the adventitial layer, which after the overnight

incubation in the RNA*later* reagent at 4ºC became easy to perform. The mesenteric artery and aorta samples were then removed from the RNA*later* reagent and kept in -80ºC until further experiments. This method has previously been shown to be able to preserve high-quality RNA and protein in tissue samples (Rodrigo *et al.*, 2002).

For measuring vascular K_{ATP} channel functional activity, thoracic aortae and the main-trunk mesentery arteries were excised and freed of connective tissue in ice-cold Krebs buffer (in mM: NaCl 120, KCl 5.9, $NaHCO_3$ 15.5, NaH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.2, and glucose 11.5). These vessels were then immediately used in the rubidium-86 ($^{86}Rb^+$) experiments as described in **2.5.2**.

2.3. Nitrite determination

Nitrate and nitrite (collectively referred to as NOx) are stable metabolites of NO, and their levels in the cell culture medium and animal plasma can serve as a surrogate marker of NO production. As nitrite in whole blood is rapidly converted to nitrate in the erythrocyte (Kelm, 1999), plasma nitrate needs to be reduced to nitrite to fully reflect NOS activity *in vivo* before measuring nitrite levels using the Griess reaction (detailed below). In contrast, in cell culture medium NO is oxidized almost completely to nitrite given the high oxygen tension in the atmosphere; therefore, the nitrate reduction step was omitted in these *in vitro* studies.

2.3.1. Reduction of serum nitrate to nitrite

The nitrate reduction steps were adapted from Tracey (Tracey *et al.*, 1995). Briefly, plasma samples were filtered through a 1.2-μm Multi-screen filter plate (Millipore, Watford, Herts). In each individual well of a 96-well plate, 6 μl of plasma was mixed with 44 μl of distilled water, 20 μl of 0.31 M potassium phosphate buffer (pH 7.5) and 10 μl each of 0.86 mM nicotinamide adenine dinucleotide phosphate, 0.11 mM flavin adenine dinucleotide and 1.0 units·ml^{-1} of nitrate reductase (derived from *Aspergillus* species, activity 0.4 units·mg^{-1}; Roche, Mannheim, Germany). Samples were then incubated for 1h at room temperature in the dark, before performing the Griess assay. Recovery of nitrate was >95% and the assay results were not significantly affected by the presence of plasma proteins.

2.3.2. Griess Reaction

The Griess reaction is based on a nitrite-dependent diazotization and azo coupling to produce a coloured azo compound in a concentration-dependent manner (Ivanov, 2004). By adding in acidified sulphanilamide and *N*-1-naphthylamine to nitrite-containing fluid, the coloured azo compound can thus be generated, of which the concentration can be measured colorimetrically.

To determine the nitrite level in the cell culture medium, RASMC were cultured in triplicate in 12-well culture plates at a density of 2×10^4 cells per well, suspended in 1 ml of DMEM/Ham's F-12 containing 0.4% FBS (low endotoxin), 50 units·ml^{-1} penicillin, and 50 μg·ml^{-1} streptomycin. At various time points, with

or without inflammatory mediators and/or 1400W, 50 µl of medium was collected and allowed to react with 50 µl each of 0.1% (w/v) sulphanilamide in 3N HCl and 0.01% (w/v) naphthylethylenediamine dihydrochloride for 10 min at room temperature in the dark. The absorbance at 560 nm was measured with a spectrophotometer (GENios, Tecan, Reading, Berks). Nitrite concentrations were determined using sodium nitrite (0.5-50 µM in cell culture medium) as a standard (Figure 2.2). All measurements were done in triplicate.

To determine the plasma NOx level, samples first went through the nitrate reduction steps, as detailed in **2.3.1**. These steps turned 6 µl of samples into 100 µl of solution, which was allowed to react with 50 µl each of sulphanilamide and naphthylethylenediamine, and the absorbance at 560 nm was then measured in the same manner as mentioned above. Nitrite concentrations were determined using sodium nitrite (2.5-250 µM) as a standard. All measurements were done in triplicate.

2.3.3. Protein determination using the Lowry method

To normalize nitrite concentration for tissue mass, the protein content of the cells was measured. Cells in each well were dissolved in 0.5% NaOH overnight at room temperature, and the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, USA) kit used to determine protein content as per manufacturer's instructions. Absorbance at 720 nm was measured by spectrophotometry (GENios, Tecan). Protein concentrations were determined using BSA (0.5-1.5 $mg \cdot ml^{-1}$) as the standard (Figure 2.3).

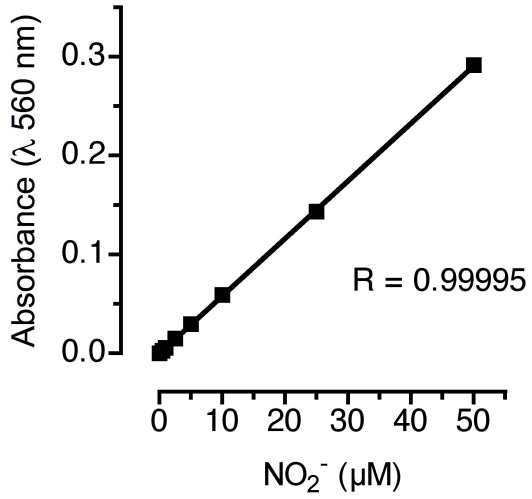


Figure 2.2. The standard curve for the Griess nitrite assay. *The nitrite standard curve was prepared by serially diluting sodium nitrite in cell culture medium to the desired concentration (*n = *3). Changes in absorbance according to nitrite concentrations fitted with linear regression.*

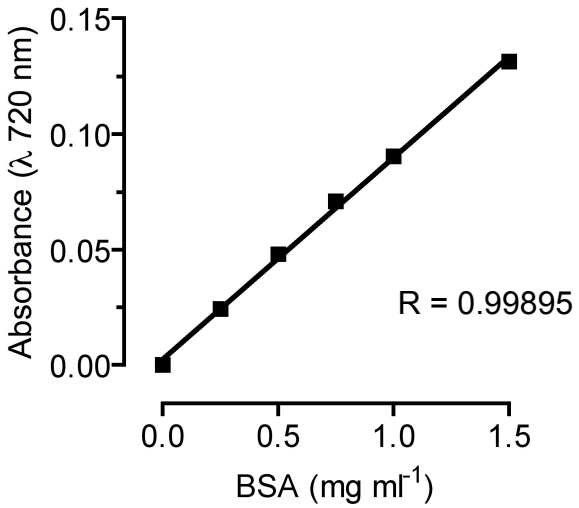


Figure 2.3. The standard curve for the BioRad protein assay. *The protein standard curve was prepared by dissolving and serially diluting bovine serum albumin (*BSA*) in 0.5% NaOH to the desired concentration (*n = *3). Changes in absorbance according to BSA concentrations fitted with linear regression.*

2.4. Molecular biology and biochemistry

2.4.1. Tissue homogenization and total RNA extraction

Frozen samples of rat aorta and mesenteric artery were pulverized into powder using a pre-chilled metal pestle and mortar. Before each pulverization the pestle and mortar were cleaned and wiped with RNase AWAY Reagents (Invitrogen, Paisley, Renfrew) to prevent cross-contamination. After pulverization, the tissue powder was carefully collected into a pre-chilled, RNase-free microcentrifuge tube, making sure the powder was not thawed.

For total RNA extraction, each sample of tissue powder was suspended in 1 ml of TRIzol reagent (Invitrogen). The suspension was then vortexed vigorously for 2 min, and then removed to a QIAshredder Spin-Column Homogenizer (Qiagen, Crawley, West Sussex). The column was centrifuged at 16,000*g* for 2 min at 4ºC in a benchtop microcentrifuge (Model 5415R, Eppendorf, Histon, Cambridge) and the flow-through removed to a pre-spun Phase Lock Gel Heavy tube (Eppendorf), into which 200 µl of chloroform was subsequently added. The tube was then manually shaken vigorously for 15 sec and then allowed to incubate at room temperature for 2-3 min; then it was centrifuged for 5 min at 12,000*g* and 4ºC to separate the phases. The aqueous phase (~450 µl in volume) was removed to an RNase-free microcentrifuge tube into which five-sixth volume (~375 µl) of propran-2-ol (Sigma-Aldrich) was added. The tube was then vortexed, and the mixture removed to an RNeasy Mini Spin Column for subsequent total RNA extraction using the RNesay Mini RNA extraction kit (Qiagen, Crawley, West Sussex), as per manufacturer's instructions (see **2.4.2**).

Using this procedure, a typical thoracic aorta and mesenteric arterial bed yielded approximately 12-18 µg and 5-8 µg of RNA, respectively.

2.4.2. Cell homogenization and total RNA and protein extraction

For total RNA extraction, RASMC were scraped off the bottom of the T-75 flask in the presence of 600 µl Buffer RLT from the RNeasy Mini kit. The cell suspension was vortexed, and then removed to a QIAshredder Spin-Column Homogenizer. The column was centrifuged at 16,000*g* in a benchtop microcentrifuge (Model 5415R, Eppendorf) for 2 min at 4ºC, and the flow-through collected for total RNA extraction using RNeasy Mini kit as per manufacturer's instructions. Briefly, ethanol was added to the flow-through to facilitate the binding of the RNA molecules to the resin in the RNeasy Mini Spin Column. Genomic DNA, small RNA and protein were subsequently washed away with two buffers. RNA of size >200 nucleotides was then eluted from the Spin Column with diethylpyrocarbonate (DEPC)-treated water.

The amount of total RNA obtained from each sample was quantified by measuring absorbance at 260 nm (A_{260}) of the RNA solution with a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). Each unit of change in the absorbance value at A_{260} compared to blank solution represents a RNA concentration of 40 ng·µl^{-1}. Absorbance at 280 nm (A_{280}) arises from contaminating proteins, and an A_{260}/A_{280} ratio >1.8 indicates adequate quality of RNA. In the current set of experiments, all samples used had an A_{260}/A_{280} ratio >1.97.

For protein extraction for Western blotting, RASMC were scraped off the bottom of the T-75 flask in the presence of 100 µl lysis buffer (in mM: Tris·HCl [pH 7.5] 50, ethylene glycol tetraacetic acid 2, DTT 0.5, phenylmethylsulphonyl fluoride 1, Na_3VO_4 1; to every 10 ml of buffer add a Complete Mini Protease Inhibitor Cocktail Tablet [Roche Biologicals, Burgess Hill, West Sussex]). The cell suspension was removed to a QIAshredder Spin-Column Homogenizer and centrifuged at 16,000*g* and 4ºC in a benchtop microcentrifuge (Model 5415R, Eppendorf) for 2 min; the flow-through was collected and kept in -80 ºC. A small aliquot was collected for protein measurement using the BCA Protein Assay (Novagen, Beeston, Nottingham) kit as per manufacturer's instructions. Absorbance at 560 nm was measured by a plate reader (GENios, Tecan). Protein concentrations were determined using BSA (20-1,000 µg·ml^{-1}) as the standard (Figure 2.4).

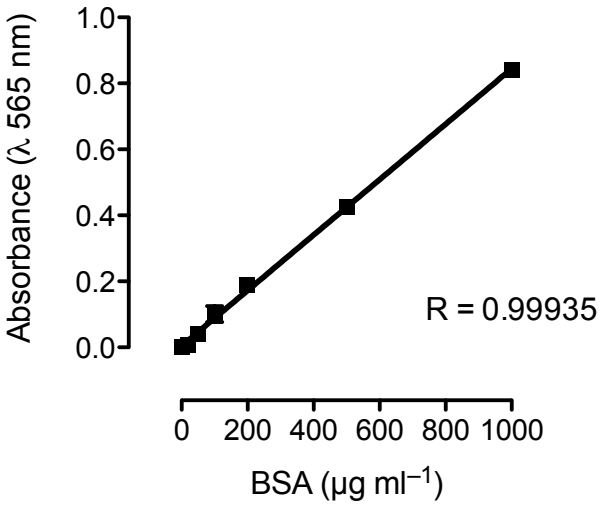


Figure 2.4. The standard curve for the BCA protein assay. *The protein standard curve was prepared by serially dilutin bovine serum albumin (*BSA*) solution to the desired concentration (*n = 3*). Changes in absorbance according to BSA concentrations fitted with linear regression.*

2.4.3. Reverse transcription into complementary DNA

For cell samples, 5 μg of RNA was used to synthesize complementary DNA (cDNA) using oligo-deoxythymidine primer (oligo-d$(T)_{12-18}$; New England Biolabs, Hitchin, Herts) and SuperScript II RT reverse transcriptase kit (Invitrogen) in a 20 μl reaction as per manufacturer's instructions. One-tenth of the product of RT reaction was used as the template for conventional PCR. For tissue samples, 3 μg RNA for aortae and 1 μg for mesenteric arteries were used to synthesize cDNA using oligo-deoxythymidine primer (oligo-d$(T)_{20}$; Invitrogen) and SuperScript III RT reverse transcriptase kit (Invitrogen) in a 20 μl reaction as per manufacturer's instruction. The cDNA products were diluted with DEPC-treated water into 1/30× solution for aorta and 1/10× solution for mesenteric artery samples; 2 μl was used for each of the triplicates as the template for quantitative PCR.

2.4.4. PCR and real-time quantitative PCR

PCR were set up as 50 μl reactions containing 0.5 unit of *Taq* DNA polymerase supplied with ThermoPol Buffer (New England Biolabs), 25 pmol of the forward and reverse primers, 10 nmol of deoxynucleoside triphosphates (dNTPs), and 2 μl of cDNA synthesized from the previous 20 μl RT reaction. The sequences of the oligonucleotide primers (Sigma-Genosys, Haverhill, Suffolk) were: K_{IR}6.1 (*Kcnj8*, accession number: NM_017099) forward 5'-CAC AAG AAC ATC CGA GAG CA-3', and reverse 5'-TGG AGA AGA GAA ACG CAG AAG-3'; SUR2B (*Abcc9*, accession number: NM_013040) forward 5'-CCA AGA ACC TCC ACC ACA A-3', and reverse 5'-TCA TCC CAA TAG CCG ACA-3'. The primer

sequences for K_{ATP} channel subunits were designed using Primer3-web software, version 0.3.0 (http://frodo.wi.mit.edu /primer3/input.htm; (Rozen and Skalesky, 2000). All primer sets are intron-spanning. PCR was performed using an automated thermal cycler (Dyad PTC-220, MJ Research, Dunmow, Essex) under the following conditions: 92ºC for 1 min 15 sec, followed by 35 cycles at 92ºC for 45 sec, 59ºC for 30 sec, and 72ºC for 1 min, and lastly by an extended elongation step of 72ºC for 10 min. PCR products were verified by electrophoresis on 2% Tris-acetic acid-EDTA agarose gel containing 1 $\mu g \cdot ml^{-1}$ ethidium bromide and visualised under an ultraviolet lamp. Electrophoretic band intensity was quantified using ImageJ software, version 1.37 (National Institutes of Health, Bethesda, ML, USA), with respect to that of the housekeeping gene, β-actin (*Actb*, accession number: NM_001101), of which the primer sequences were forward 5'-GGC TAC AGC TTC ACC ACC AC-3', and reverse 5'-TAC TCC TGC TTG CTG ATC CAC-3'. The amount of RT product in β-actin lanes was reduced to one-fourth, *i.e.* 0.5 µl of cDNA synthesized from the previous 20-µl RT reaction was used to generate comparable signal intensity so as to facilitate comparison with bands of K_{ATP} subunit mRNA. The predicted sizes of the amplicons are: 265 base pairs (bp) for $K_{IR}6.1$, 189 bp for SUR2B, and 497 bp for β-actin.

Real-time quantitative PCR (Heid *et al.*, 1996) were set up in triplicate in white 96-well plates (ABgene, Epsom, Surrey) as 20 µl reactions containing 10 µl of SYBR Green PCR Master Mix (Applied Biosystems, Warrington, Cheshire), cDNA from the previous RT reaction in the amount as described in **2.4.3**, and the forward and reverse primers, of which the amounts are detailed below.

SYBR Green PCR Master Mix contains SYBR Green I dye, hot start AmpliTaq Gold DNA polymerase, dNTPs, and optimized buffer components. SYBR Green I is an asymmetrical cyanine dye that binds to double-stranded DNA (dsDNA). The resulting DNA-dye complex absorbs blue light (λ_{max} = 497 nm) and emits green light (λ_{max} = 522 nm), which is detected by the real-time PCR machine to measure the amount of dsDNA. At the concentrations used for real-time PCR,

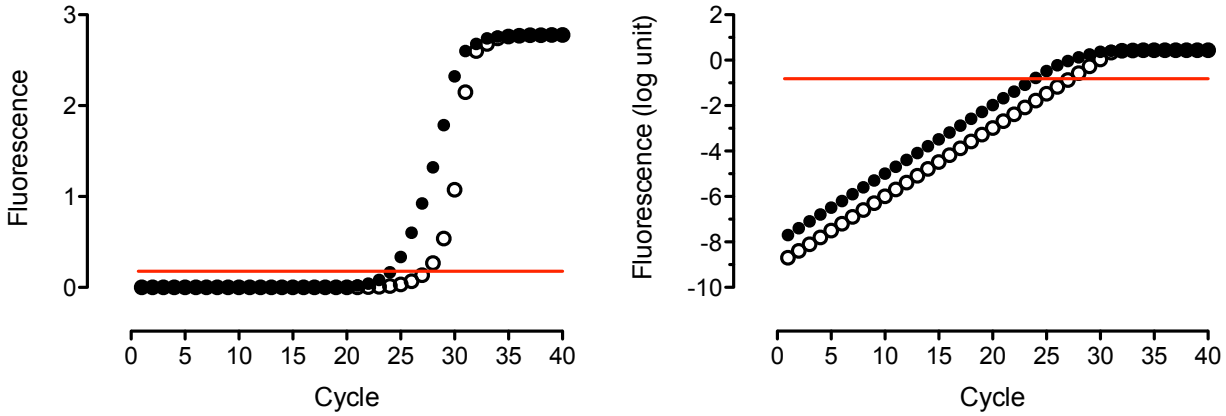
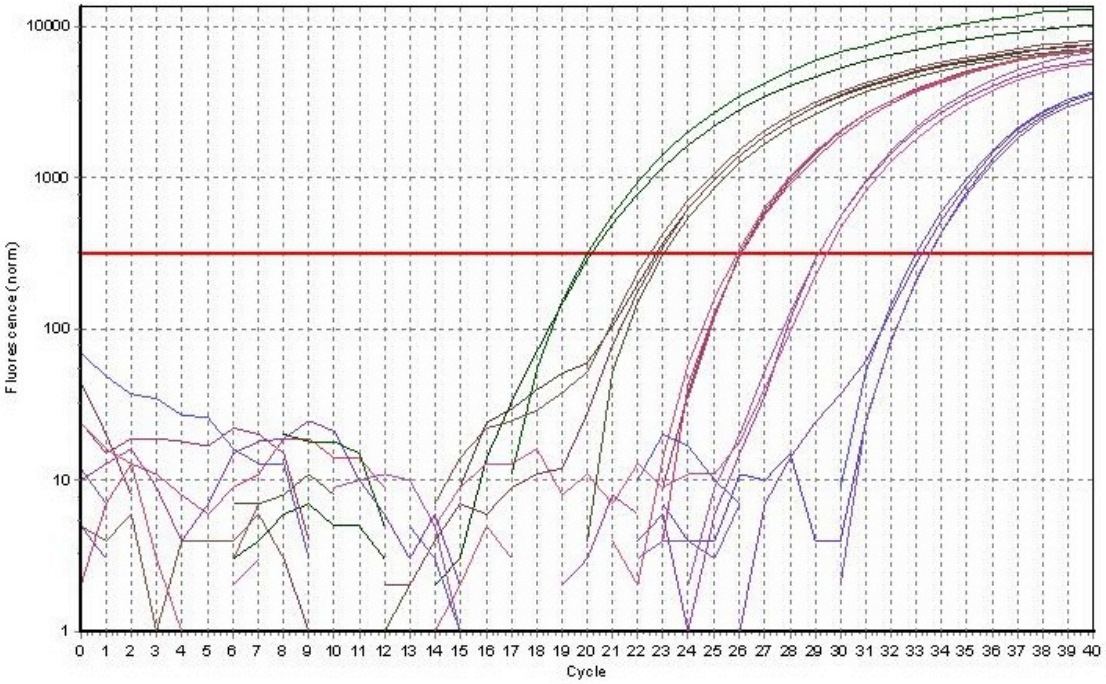


Figure 2.5. Determination of the threshold cycle (C_T) value in a real-time polymerase chain reaction (PCR). *Figures were generated from mathematical simulation. It assumes the presence of 10^9 copies of double-stranded DNA binding to SYBR Green I dye emits a fluorescence intensity of 1 unit and an amplification efficiency of the PCR to be 100%. The exponential increase in PCR products (the linear portion of the curves in the right panel, which is the same as the left panel except the y axis is of log scale) will not be detected until the accumulated PCR product binding to SYBR Green I reaches the amount that can emit fluorescence beyond the threshold level (indicated by the red lines). For a reaction that begins with 10 copies of template (closed circle), this event happens at cycle 24 (i.e., C_T = 24.0), whereas for a reaction that begins with 1 copy of template (open circle), it happens between cycles 27 and 28 (i.e., C_T = 27.3). C_T values can thus represent the template number in a given sample (Heid* et al.*, 1996). Threshold is often automatically determined by the real-time PCR signal detection software, which often uses the fluorescence intensity 10 times the standard deviation above the noise of the baseline.*

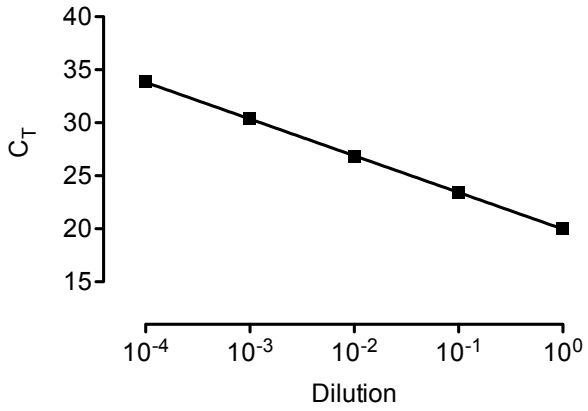
free SYBR Green I or that bound to the single-stranded DNA emits very low background fluorescence, whereas SYBR Green I bound to dsDNA emits much stronger fluorescence of approximately 2,000 times the initial, unbound fluorescent signal when properly illuminated (Shipley, 2006). Real-time PCR reports the threshold cycle (C_T) value as the quantitative measurement of the initial template amount in a reaction (Heid *et al.*, 1996). Briefly, the lower the C_T value, the more copies of template in the sample assayed (Figure 2.5). In RT-PCR, it represents an increased transcriptional expression of the gene of interest yielding more mRNA.

The sequences of the oligonucleotide primers (Sigma-Genosys) designed using Primer3-web software, version 0.3.0, were: K_{IR}6.1 forward 5'-CGT CAC ACG CTG GTC ATC TTC -3', and reverse 5'-TCA GTC ATC ATT CTC CCT CCA AAC C-3'; SUR2B forward 5'-GCG GAT CGC ACG GTT GT-3', and reverse 5'-CGA ACG AGG CGA ACA CTC CA-3'; and the reference gene, hydroxymethylbilane synthase (*Hmbs*, accession number: NM_013168) forward 5'- CAC CTG GAA GGA GGC TGT AG-3', and reverse 5'-CAG GTA CAG TTG CCC ATC CT-3'. All primer sets were intron-spanning. *Hmbs* was chosen to be the reference gene in this study as its expression appears to be unaltered by sepsis (Bilbault *et al.*, 2004; Alamdari *et al.*, 2008). The predicted sizes of the amplicons are: 242 bp for K_{IR}6.1, 148 bp for SUR2B, and 69 bp for Hmbs. The annealing temperature and primer concentrations were optimized, and the calibration curve and amplification efficiency for each pair of primers were determined (Figure 2.6). Eight pmol of each primer is used in each reaction except for the reverse primers of K_{IR}6.1 and Hmbs, for which 10 pmol was used.

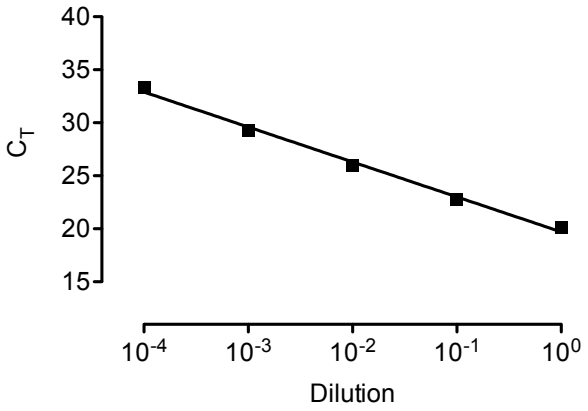
Figure 2.6. Calibration curves and assay efficiency (E) of the real-time polymerase chain reaction. *The calibration curves were constructed using serial dilutions of cDNA and their corresponding C_T value determined. The assay efficiency is calculated based on the slope of respective curves: Efficiency = $[10^{(-1/slope)}] - 1$.*



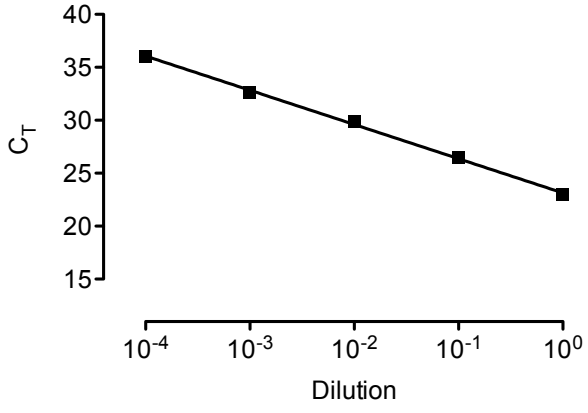
$K_{IR}6.1$ (E = 1.945; R = 0.99991)



SUR2B (E = 2.011; R = 0.99730)



Hmbs (E = 2.039; R = 0.99917)



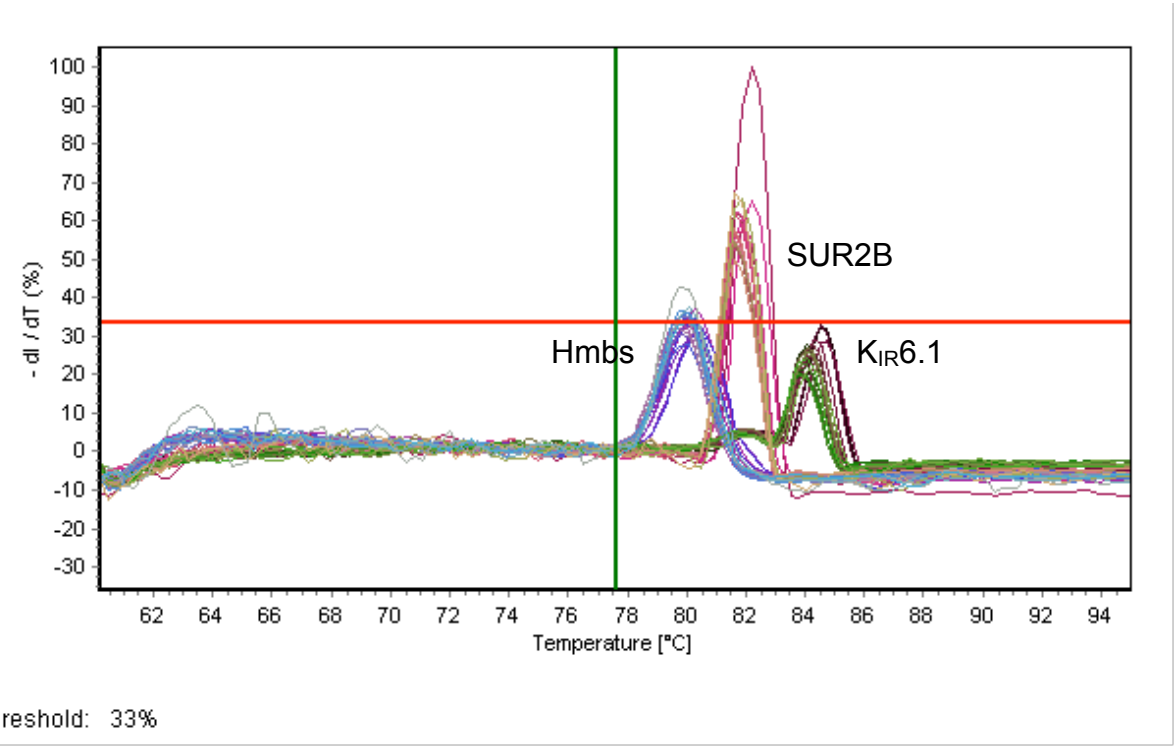


Figure 2.7. Melting curve analysis of the real-time polymerase chain reaction (PCR) amplicons. *A double-stranded DNA (dsDNA) molecule dissociates into two single-stranded DNA molecules at a specific temperature depending on its length and guanine-cytosine content. Through heating up the PCR product at a constant rate of increasing temperature and detecting the point of abrupt decrease in fluorescence emission (indicating massive dissociation of dsDNA molecules), the melting temperature can be determined. The figure showed the melting curves of 8 samples, each assayed in triplicate. The single peak for amplicons from each primer pair indicates that no primer-dimers were synthesized in the experiment.*

Real-time quantitative PCR was performed using an automated thermal cycler (Mastercycler ep realplex, Eppendorf) under the following conditions: 94ºC for 10 min, followed by 40 cycles at 94ºC for 15 sec, 60ºC for 2 sec, and 72ºC for 30 sec, followed by a melting curve analysis consisting of a linear increase in temperature from 60ºC to 95ºC in 20 min (Figure 2.7).The relative amount of the cDNA in relation to the reference gene (which normalizes the amount of template input) is calculated using the 'delta-delta C_T' method corrected with

primer efficiency (Souazé *et al.*, 1996). Briefly, the ratio of the cDNA amount of the target and the reference genes can be calculated using the equation:

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta C_{T\,\text{target}}(\text{control}-\text{sample})}}{(E_{\text{Ref}})^{\Delta C_{T\,\text{Ref}}(\text{control}-\text{sample})}}$$

E_{target} = assay efficiency of the target amplicon

E_{Ref} = assay efficiency of the reference amplicon

$\Delta C_{Ttarget}$(control – sample) = average C_T of target amplicon in the control group – C_T of target amplicon in individual samples

ΔC_{TRef}(control – sample) = average C_T of reference amplicon in the control group – C_T of reference amplicon in individual samples

Equation 2.2. Relative quantitation of the target amplicon with respect to the reference amplicon in a real-time polymerase chain reaction.

Thus in this study, the ratio of K_{IR}6.1/Hmbs and SUR2B/Hmbs cDNA amount can be calculated in both sham and septic animals. Provided the amount of reference gene cDNA is not altered by the experimental condition, *i.e.* in the current study *Hmbs* expression was not altered by the septic stimulus, the ratio of K_{IR}6.1 and SUR2B cDNA amount between sham and septic animals can thus be calculated (Figure 2.8).

PCR products were verified by electrophoresis on 2% Tris-acetic acid-EDTA agarose gel containing 1 $\mu g \cdot ml^{-1}$ ethidium bromide, and visualized under an ultraviolet lamp. The PCR amplicons were submitted for DNA sequencing (Scientific Support Services, Wolfson Institute for Biomedical Research, UCL) to ensure correct product generation.

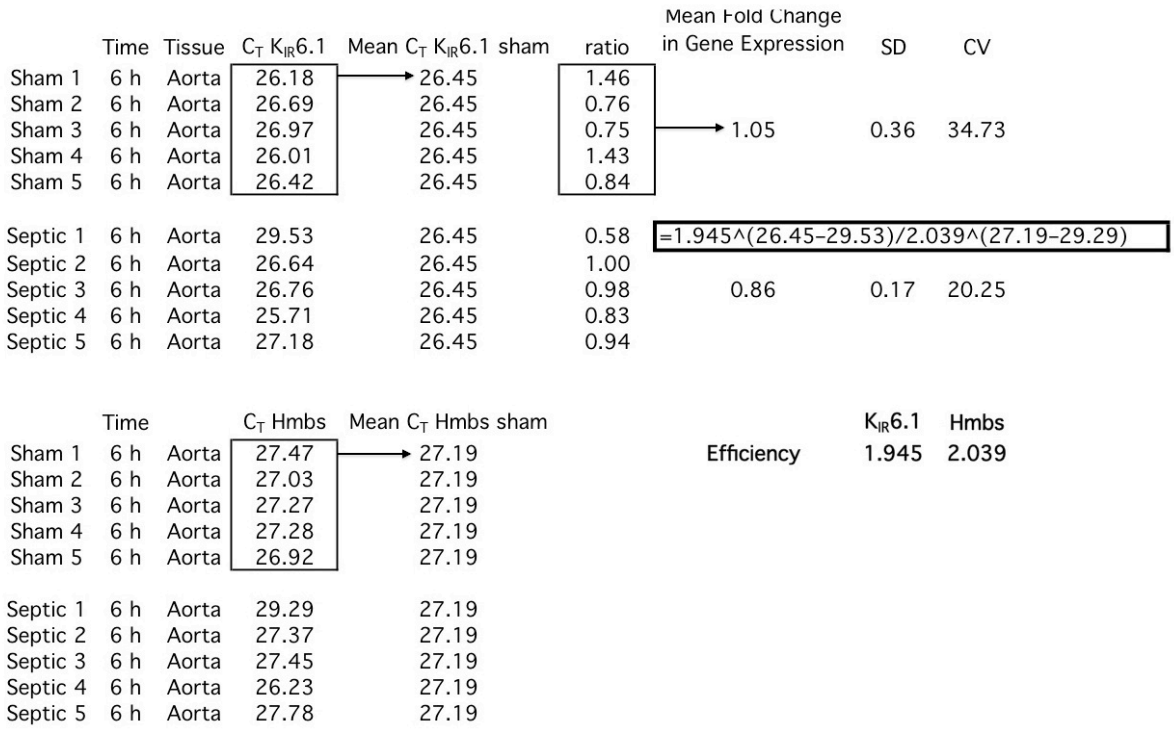


Figure 2.8. Calculation of the mean fold change in K_{IR}6.1 gene expression in aortae of sham and septic rats at 6h. *Modified from Livak and Schmittgen (2001).*

2.4.5. Western blotting

7.5% sodium dodecyl sulphate (SDS)-polyacrylamide gels were prepared and poured following standard recipes: stacking gel (7.5% acrylamide [Bio-Rad], 0.1% (w/v) SDS [Fluka], 125 mM Tris [pH 6.8], 0.05% (w/v) ammonium persulphate, and 0.1% (v/v) *N,N,N',N'*-tetramethylethylenediamine (TEMED)); resolving gel (7.5% acrylamide, 0.1% (w/v) SDS, 375 mM Tris [pH 8.8], 0.05% (w/v) ammonium persulphate, and 0.05% (v/v) TEMED). Prior to gel loading, cell homogenates were diluted in 6× loading buffer with a final concentration of 1.7% (w/v) SDS, 58.3 mM Tris (pH 6.8), 100 mM DTT, and 5% (w/v) glycerol. Sample were mixed and heated to 95ºC for 15 min to denature proteins. Gels

were run in a standard running buffer containing 0.1% SDS, 25 mM Tris and 192 mM glycine. Samples were fractionated at constant currents of 20 (in stacking gels) and 30 (in resolving gel) mA for every 1.5 mm-thick gel for 45 min until the dye front had run off the bottom of the gel. The gel was subsequently loaded onto a blotting apparatus and sample transferred to the polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Watford, Herts) in a transfer buffer containing 10 mM 3-(cyclohexylamino)-1-proprane sulphonic acid (CAPS; pH 11) and 10% methanol. The gel was allowed to transfer at constant voltage of 50 volts at room temperature for 30 min, and then the membrane was air-dried for 15 min after soaking in 100% methanol for 10 sec. The reason for using CAPS buffer instead of the routinely used Tris-glycine buffer is the possible high isoelectric point (pI) of the $K_{IR}6.1$ protein. The computed theoretical pI is 9.35, calculated using the "Compute pI/Mw tool" website calculator (http://www.expasy.ch/tools/pi_tool.html; (Bjellqvist *et al.*, 1994) of the Swiss Institute of Bioinformatics' ExPASy Proteomics Server (Gasteiger *et al.*, 2003). The routinely used Tris-glycine buffer (pH 8.3) causes the $K_{IR}6.1$ protein to migrate toward the opposite direction to where the PVDF membrane is, thereby resulting in failure of blotting and protein loss.

For Western blotting, the Amersham ECL detection kit (GE Healthcare, Little Chalfont, Bucks) was used. Briefly, the air-dried PVDF membrane was wetted by soaking in 10% methanol for 15 sec and then in Milli-Q water (Millipore) for 2 min. It was then incubated at room temperature for 60 min in the blocking agent that consists of 10% non-fat dried milk dissolved in Tris-buffered saline (100 mM Tris·Cl [pH 7.5] in 0.9% (w/v) NaCl) containing 0.1% (w/v) Tween 20 (TBS-

T). The primary antibody, either goat anti-K_{IR}6.1 (R-14) (diluted 1:500) or goat anti-SUR-2B (C-15) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (diluted 1:200) was added into the blocking agent and incubated with the blot for 60 min at room temperature with agitation. This was followed by 3 × 15 min washes with TBS-T. The secondary antibody (horseradish peroxidase-conjugated rabbit anti-goat IgG; Santa Cruz) was diluted 1:10,000 in the blocking agent and incubated with the blot for a further 60 min at room temperature with agitation. This was again followed by 3 × 15 min washes with TBS-T. The detection reagent was constituted through mixing equal volumes of detection solution 1 with detection solution 2. The reagent was then applied to the surface of the membrane, and the blots incubated for 1 min at room temperature. Excessive detection regent was then drained, and the fluorescence signals detected using autoradiography film.

After signal detection, the same PVDF membrane was stained with Coomassie brilliant blue R (Bio-Rad) as per manufacturer's instructions to normalize the quantity of protein loaded. Briefly, the membrane was incubated in a solution of 0.1% Coomassie brilliant blue R in 50% methanol and 7% acetic acid for 2 min. It was de-stained in 50% methanol and 7% acetic acid for 10 min. The third step of de-staining in 90% methanol and 10% acetic acid for 10 min was not performed as this often leads to the excessive de-staining of the membrane.

2.5. Vascular K_{ATP} channel functional activity

2.5.1. Membrane potential dye assay

K_{ATP} channel functional activity was assessed using the membrane potential-sensitive fluorescence dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$; Molecular Probes, Paisley, Renfrew) following a published protocol (Gopalakrishnan *et al.*, 1999). $DiBAC_4(3)$ enters depolarized cells where it binds to intracellular proteins or membranes and exhibits enhanced fluorescence. Increased depolarization results in more influx of the anionic dye, leading to an increase in fluorescence. Hyperpolarization results in extrusion of the dye and a decrease in fluorescence (Apell and Bersch, 1987). Briefly, RASMC were plated out onto 96-well black clear-bottomed plates at a density of 2×10^4 cells per well using a total volume of 200 µl, and were grown in DMEM/Ham's F-12 supplemented with 10% FBS, 50 $U \cdot ml^{-1}$ penicillin and 50 $\mu g \cdot ml^{-1}$ streptomycin. Upon reaching confluence on about Day 3 or 4, cells were serum-starved for 48h before being treated with LPS and IL-1β for 48h.

Upon treatment completion, cells were rinsed twice with 200 µl of assay buffer (in mM: NaCl 120, KCl 2, $CaCl_2$ 2, $MgCl_2$ 1, glucose 5, HEPES 20, tetraethylammonium [TEA] 1, pH 7.4 at 25°C) containing 5 µM $DiBAC_4(3)$. TEA was added at this concentration so as to inhibit BK_{Ca} channel activity, a major K^+ conductance in smooth muscle (Clapp and Gurney, 1991; Clapp and Tinker, 1998). Following this procedure, cells were incubated with 180 µl of the same $DiBAC_4(3)$-containing buffer for at least 30 min in a cell incubator at 37ºC and 95% air/5% CO_2. The 96-well plates were then moved to a fluorescent imaging

plate reader (FLIPR; Schroeder and Neagle, 1996; Molecular Devices, Paisley, Renfrew). Assays were carried out at 37ºC, being initiated by addition of 20 µl of a 10× concentration of Lev at different test concentrations prepared in the assay buffer containing 5 µM $DiBAC_4(3)$. Changes in fluorescence were monitored for 25 min by sampling every 30 sec from 96 wells simultaneously, at excitation and emission wavelengths of 488 and 520 nm, respectively. The absolute value of the maximal levcromakalim-evoked change in fluorescence (ΔF) for each well was normalized to the background fluorescence (F). Changes in fluorescence are thus shown as $\Delta F/F$.

2.5.2. Rubidium efflux

The radioisotope rubidium-86 ($^{86}Rb^+$) has been widely used in many tissues as a surrogate marker for K^+ efflux/loss from cells (*e.g.* Bolton and Clapp, 1984). It is more convenient to use than $^{42}K^+$ due to its much longer half-life (19 days versus 12h). Aortae and main trunk mesenteric arteries preserved in ice-cold Kreb's buffer (see **2.2.3**) were carefully cut into two pieces along their longitudinal axis. The endothelium was gently removed through rubbing with a cotton swab. The arterial strips were then incubated in DMEM/Ham's F-12 containing $^{86}Rb^+$ (0.037 $MBq \cdot ml^{-1}$) for 3h to reach equilibrium of loading (Bolton and Clapp, 1984). The loading medium was then removed, and the arterial strips washed three times with assay buffer (in mM: NaCl 136.8, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, $MgSO_4$ 0.4, $NaHCO_3$ 2.5, NaH_2PO_4 0.65, KH_2PO_4 0.44, glucose 5.5, HEPES 7.5, TEA 1) to remove excess $^{86}Rb^+$. Each arterial strip was then placed in a well of a 6-well plate, and 2 ml of assay buffer added to each well.

One ml of buffer was immediately collected to determine the background value, and the other ml was removed 15 min later to determine the amount of baseline $^{86}Rb^{+}$ efflux. Another 1 ml of assay buffer containing 10 μM of levcromakalim was then added to each well and removed 15 min later to determine the $^{86}Rb^{+}$ efflux in response to vascular K_{ATP} channel opening. The amount of radioactivity in each sample/supernatant was measured by Packard 2000CA Tri-Carb liquid scintillation analyzer (Packard Instruments, Cambridge, Cambridge) and normalized to the total amount in the blood vessel at that time.

2.6. Chemicals

All *in vitro* drugs were prepared and diluted in water, unless otherwise specified. Levcromakalim for use *in vitro* and $DiBAC_4(3)$ were dissolved in dimethyl sulphoxide (DMSO). The final concentration of DMSO in *in vitro* experimental solutions did not exceed 0.2% (v/v). All chemicals were purchased from Sigma-Aldrich, except the following: IL-1β was purchased from R&D Systems; midazolam (midazolam hydrochloride) was from Roche (Welwyn Garden City, Herts); NE (norepinephrine tartrate) was from Abbott Labs; pentolinium was from Acros Organics (Morris Plains, NJ, USA); and PNU-37883A was from BIOMOL International (Exeter, Devon). All chemicals were of highest grade available.

2.7. Statistical analysis

The number of experiments was chosen on the basis of previous similar experiments conducted in my host lab. A formal power calculation, from which the sample size needed to show statistically and clinically significant differences was, however, not performed. All figures are presented as mean ± standard error of the mean (S.E.M.) of *n* observations. Statistical analysis between two groups was performed using paired or unpaired Student's *t* test, and between multiple groups using one-way analysis of variance (ANOVA). *In vivo* arterial pressures were compared between time-points and clinical groups using two-way ANOVA (with repeated measures where appropriate). ANOVA values were corrected for comparisons against the control group (Bonferroni *post-hoc* test) or all groups (Newman-Keuls *post-hoc* test) using Prism 5.0 (GraphPad Software, La Jolla, CA, USA). In all analyses, a p value <0.05 was considered statistically significant.

Chapter Three Vascular K_{ATP} channel expression

3.1. Introduction

I began the project with an investigation of whether vascular K_{ATP} channel gene expression was influenced by septic stimuli, hypothesizing that the channel gene could be induced during sepsis, leading to an increase in channel number and thus channel activity. (Czaika *et al.*, 2000) showed an increased expression of the pore-forming $K_{IR}6.1$ subunit in the diaphragm of the endotoxemic rat. The authors did not assessed the change in channel activity. Later, in a model of murine experimental colitis, an increased transcriptional expression of $K_{IR}6.1$ in colonic smooth muscle was associated with an increase in the whole cell current both basally and when stimulated by the K_{ATP} channel opener, levcromakalim (Jin *et al.*, 2004). More recently, Shi *et al.* (2010) showed that murine aortae exposed *in vitro* to LPS (1 $\mu g \cdot ml^{-1}$) had an increase in both $K_{IR}6.1$ and SUR2B gene expression. This was accompanied by an increase in basal K_{ATP} current density. Thus sepsis may be able to induce an increase in channel gene expression that ultimately augments channel functional activity. However, to which extent does channel gene induction contribute to the increase in channel functional activity remains to be determined, as the former does not necessarily result in the composition of functioning channels (Crane and Aguilar-Bryan, 2004). Whether channel gene induction also appears *in vivo* also awaits confirmation.

3.2. K_{ATP} channel expression and activity in RASMC

3.2.1. Cell viability

Using the trypan blue dye exclusion method, the viability of RASMC subjected to a 48h treatment with LPS (1 $\mu g \cdot ml^{-1}$) and IL-1β (10 $ng \cdot ml^{-1}$) was found to be similar (97.8 ± 1.6%, *n* = 3) to control cells (97.5 ± 1.7%, *n* = 3; *p* = NS). Thus treatment itself did not appear cytotoxic or detrimental to cell survival.

3.2.2. Nitrite levels in culture medium

I first sought to determine if treatment of RASMC with a combination of LPS and IL-1β could induce NOS2 and generate excessive amounts of NO. In control RASMC, the nitrite level in the culture medium was 0.3 ± 0.2 μM at 24h, which rose to 1.0 ± 0.3 μM at 48h, though not significantly (*n* = 4). Upon treatment with LPS and IL-1β, the nitrite level profoundly increased ~30-fold to 9.2 ± 1.3 μM at 24h, and ~15-fold to 15.4 ± 1.7 μM at 48h (*p* <0.01; Figure 3.1). For all subsequent experiments, I chose to incubate cells for 48h with inflammatory mediators, since the NO levels were substantially higher at this time point compared to 24h.

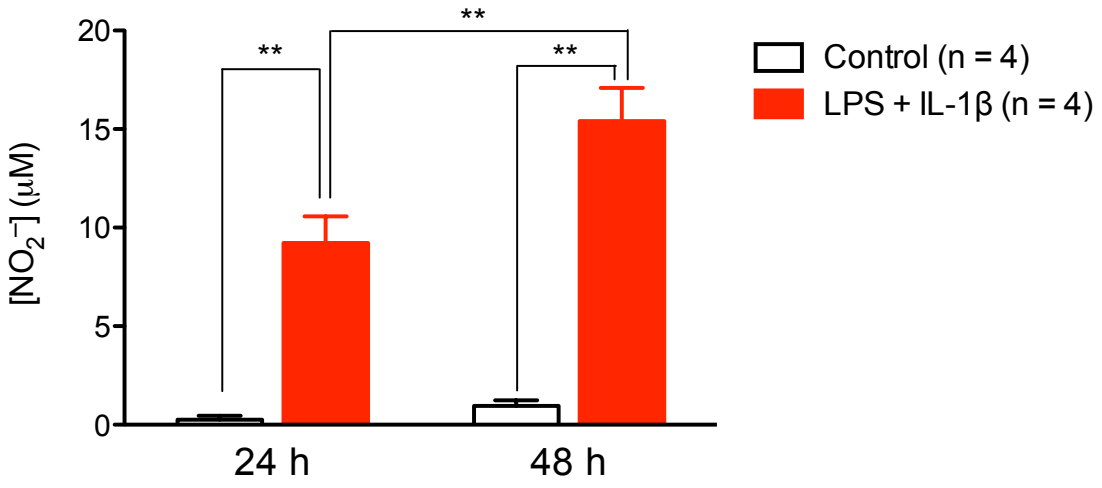


Figure 3.1. Effect of inflammatory mediators on nitrite production by cultured rat aortic smooth muscle cells (RASMC). *Nitrite levels in the culture medium were measured following treatment of RASMC with lipopolysaccharide (*LPS*; 1 μg·ml^{-1}) and interleukin (*IL*)-1β (10 ng·ml^{-1}). Cells were serum-starved for 48h in culture medium before LPS and IL-1β were added for both 24 or 48h.* **p *<0.01 (two-way ANOVA with Bonferroni* post-hoc *test).*

Figure 3.2 shows nitrite levels in the cell culture medium harvested from cells receiving a septic stimulus with or without pre-treatment with the NOS2-selective inhibitor, 1400W (10 μM). For control cells, the nitrite level was 1.8 ± 0.2 μM·mg^{-1} protein (*n* = 6), and for 1400W-treated cells 2.6 ± 0.3 μM·mg^{-1} protein (*n* = 4; *p* = NS vs. control). Nitrite levels in the cell culture medium were significantly higher for cells treated with LPS and IL-1β compared to control (34.9 ± 5.6 μM·mg^{-1} protein; *n* = 6; *p* <0.01), though no such increase (*n* = 4; *p* = NS vs. control) was observed in cells pre-treated with 1400W.

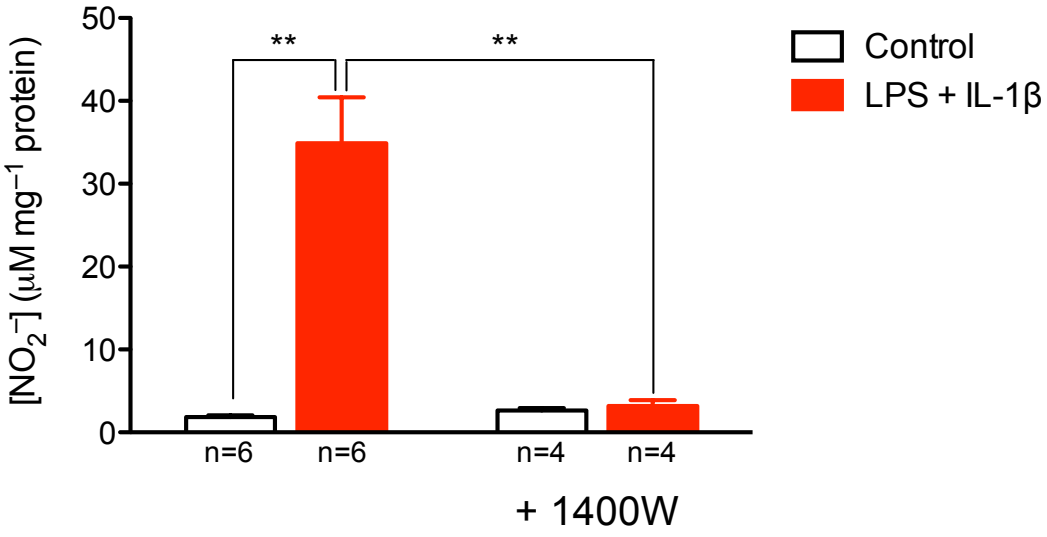


Figure 3.2. Effect of inflammatory mediators and the NOS2 inhibitor 1400W on nitrite production by cultured rat aortic smooth muscle cells (RASMC). *Nitrite levels in culture medium harvested from quiescent RASMC incubated with lipopolysaccharide (*LPS;*1 μg·ml^{-1}) and interleukin (*IL*)-1β (10 ng·ml^{-1}) for 48h, with or without pre-treatment of 1400W (10 μM) were measured and normalized to cell protein.* **p <*0.01 (two-way ANOVA with Bonferroni* post-hoc *test).*

In summary, the dosage of 1 μg·ml^{-1} of LPS and 10 ng·ml^{-1} of IL-1β for 24 to 48h induced RASMC to generate substantial amounts of NO, as determined by several fold increases in nitrite levels. 1400W effectively inhibited NO generation, suggesting NO was synthesized by NOS2 in this model. RASMC exposed to LPS and IL-1β at the above concentrations for 48h showed no change in cell viability.

3.2.3. RT-PCR

I next wanted to establish whether the combination of LPS and IL-1β would change the transcriptional levels of K_{ATP} subunit genes. I firstly designed PCR primer sets and optimized the thermal cycle protocol and cycle number. I then tested the primers on control RASMC, and finally the treated these cells to see if I could detect differences in K_{ATP} subunit gene transcription. Representative results of RT-PCR from serum-starved RASMC cells in the absence and presence of inflammatory mediators are shown in Figure 3.3. In LPS and IL-1β treated RASMC there were higher levels of $K_{IR}6.1$ subunit expression compared to untreated cells. When corrected for β-actin expression, the electrophoretic band intensity for $K_{IR}6.1$ was 6.6-fold higher than that of control (p <0.05, n = 5; Figure 3.4).

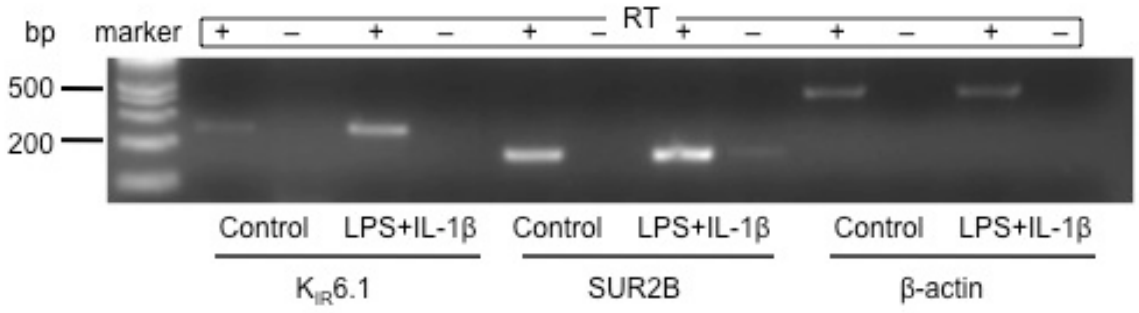


Figure 3.3. Inflammatory mediators increase expression of $K_{IR}6.1$ in rat aortic smooth muscle cells (RASMC). *Reverse transcription polymerase chain reaction analysis of $K_{IR}6.1$ and SUR2B subunits from mRNA isolated from RASMC treated using the protocol described in* ***2.1.3****. Amplified fragments (35 cycles) were electrophoresed on a 2% agarose gel. Lane one contains base pair markers and subsequent odd number lanes contain no reverse transcriptase. Visible products were detected for $K_{IR}6.1$ (lanes 2 and 4; 265 bp), SUR2B (lanes 6 and 8; 189 bp) and the housekeeping gene β-actin (lanes 10 and 12; 497 bp).*

As iNOS activity appears important in mounting inflammatory responses and sustaining the NF-κB signaling pathway (Hierholzer *et al.*, 1998; van't Hof *et al.*, 2000), it may also contribute to the enhanced $K_{IR}6.1$ gene expression in the current experiment. I therefore measured the amount of subunit mRNA in cells pre-treated with 1400W. The enhanced $K_{IR}6.1$ expression induced by a combination of LPS and IL-1β was largely reversed by 1400W (band intensity 187 ± 47%, p = NS vs. control, n = 4). In contrast, there was no change in SUR2B band intensity with the inflammatory mediators (97 ± 7%, n = 5), nor did 1400W have any effect on SUR2B expression either (n = 4, p = NS).

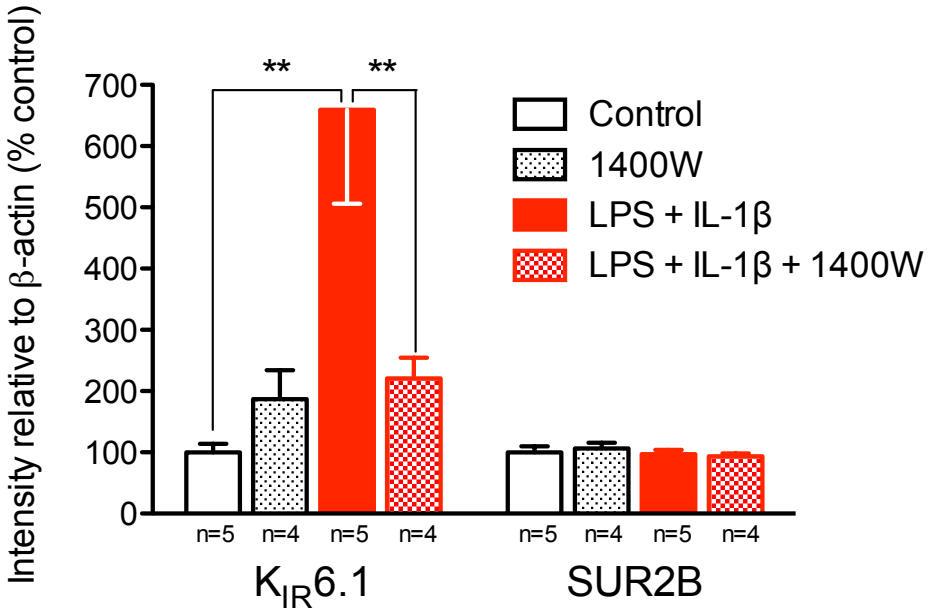


Figure 3.4. Electrophoretic band intensity of reverse transcription polymerase chain reaction amplicons of K_{ATP} channel subunits. Band intensity was normalized to the housekeeping gene, β-actin, and was referenced to that of control cells. *Quiescent RASMC were either left untreated or incubated with lipopolysaccharide (*LPS*; 1 μg·ml^{-1}) and interleukin (*IL*)-1β (10 ng·ml^{-1}) for 48h, without or with the selective iNOS inhibitor, 1400W (10 μM). 1400W was given since 1h before and remained throughout the whole period of LPS and IL-1β treatment. **p <0.01 (two-way ANOVA with Bonferroni* post-hoc *test).*

Finally, the RT-PCR amplicons corresponding to $K_{IR}6.1$ and SUR2B were cut out of the gel for sequencing (Scientific Support Services, WIBR, UCL) and were found to be identical with the reference sequences ($K_{IR}6.1$ mRNA RefSeq accession number: NM_017099; SUR2B mRNA RefSeq accession number: NM_013040).

3.2.4. K_{ATP} channel functional activity

I then investigated whether changes in K_{ATP} functional activity corresponded to changes in K_{ATP} subunit gene expression. I used the membrane potential-sensitive fluorescence dye $DiBAC_4(3)$ to assess the response of treated RASMC to the K_{ATP} channel opener, levcromakalim (Lev). More K_{ATP} channels in the plasma membrane ought to represent a larger change in $\Delta F/F$ at a given concentration of Lev.

Lev caused a decrease in fluorescent intensity (Figure 3.5**a**), consistent with the agent causing membrane hyperpolarization (Gopalakrishnan *et al.*, 1999). However, the changes in fluorescent intensity were not readily apparent until the concentration of Lev in the assay buffer reached 1 μM and above (Figure 3.5**b**). Compared to control cells, significantly larger changes in fluorescence intensity were induced by Lev at 1 to 3 μM in RASMC subject to septic stimuli. Conversely, for 1400W pre-treated cells, the effect of septic stimuli was reversed (Figure 3.5**a** and **b**). When RASMC were stimulated by 3 μM Lev, the change in fluorescence (5,968 ± 447 fluorescent units for control, and 8,211 ± 547 units for LPS and IL-1β-stimulated cells; $n = 4$, $p < 0.05$) was compatible

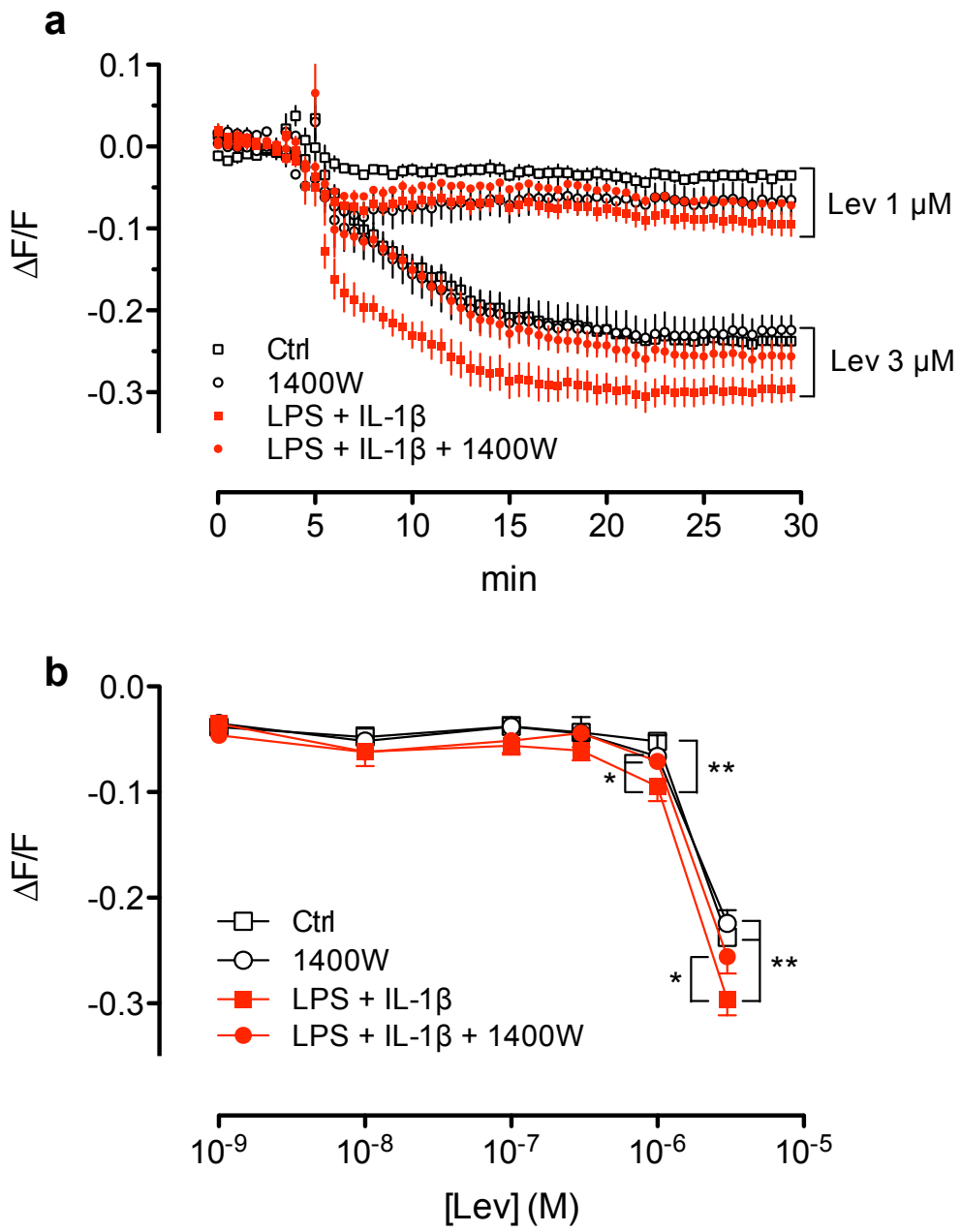


Figure 3.5. Changes in fluorescent intensity in response to levcromakalim (Lev) for rat aortic smooth muscle cells incubated with lipopolysaccharide (LPS; 1 μg·ml^{-1}) and interleukin (IL)-1β (10 ng·ml^{-1}) for 48h, with or without pre-treatment of the NOS2-selective inhibitor, 1400W (10 μM). *Changes in fluorescent intensity (ΔF) were normalized to background fluorescence (F).* ***a)*** *time-dependent changes in fluorescent intensity. Lev was added at 5 min (*n = *4);* ***b)*** *changes in fluorescent intensity at increasing concentrations of Lev in assay buffer. The final 3 readings (at 30 min) of each experiment were averaged to represent the ΔF/F of the experiment.* n = *4;* *p <*0.05,* **p <*0.01 (two-way ANOVA with Bonferroni* post-hoc *test).*

with that found in bladder smooth muscle cells (Gopalakrishnan *et al.*, 1999), but the ΔF/F in RASMC seemed less (1,562 ± 34 fluorescent units for control, and 2,755 ± 312 units for LPS and IL-1β-stimulated cells; $n = 4$, $p < 0.05$) compared to bladder smooth muscle cells (in the vicinity of 4,500 fluorescent units) when stimulated by 1 μM Lev. I did not derive the EC_{50} value in current study because higher doses of Lev were not tested and thus it is not clear that changes in ΔF/F had reached a maximum level. Nevertheless, it would surely be higher than the 0.4 ± 0.01 μM reported by Gopalakrishnan *et al.* (1999).

3.3. K_{ATP} channel expression and activity in *ex vivo* arterial blood vessels from rats with peritonitis

After establishing presence of increased K_{ATP} subunit gene expression in RASMC receiving septic stimuli and the corresponding increase in K_{ATP} channel activity, I continued to survey if the same phenomenon also occurred *in vivo*. I used a rat model of faecal peritonitis that has been well characterized in the host lab and mimics many of the clinical features of sepsis (Brealey *et al.*, 2004; Barrett *et al.*, 2007a). Further demonstration of its characteristics, including NO production, evidence of hypoperfusion, and effects of fluid resuscitation will be provided in the next chapter. Using this experimental model I collected aortae and mesenteric arteries and measured levels of K_{ATP} subunit gene expression using quantitative RT-PCR.

I had to overcome a number of hurdles in order to successfully carry out the quantitative RT-PCR experiments, which proved time consuming. Firstly, to

avoid mRNA degradation from delayed or inappropriate tissue preservation, I initially chose to snap-freeze aortae in liquid nitrogen immediately after the animal was sacrificed. To do so, I needed to free the artery from the surrounding tissue *in situ*. This proved not too difficult if the vessel in question was the thoracic aorta. However, when it came to the mesenteric artery, it was impossible to complete the task in reasonably short time unless I only cut off and collected the main trunk. This limited mass of the mesenteric artery could only yield a small amount of RNA, and its ability to represent a resistance artery is called into question. Moreover, with snap-freezing method, I could not separate the media layer of aorta from the adventitial layer, which may confound the result because this layer may also expresses K_{ATP} channels. However, this method was later substituted by one utilising the RNA*later* reagent which is capable of preserving high-quality RNA (Rodrigo *et al.*, 2002). It proved invaluable because it allowed the collection of a greater mass of the mesenteric arterial bed, as well as the isolation of the media layer of aortae.

Secondly, the extraction of RNA from aortic smooth muscle was problematic. I encountered difficulties in tissue homogenization and cell disruption, and therefore in the first instance could not obtain adequate amount of RNA from aortic samples. In my hand, the commercial kit (RNeasy Fibrous Tissue Mini kit, Qiagen) that recruits proteinase K to digest contractile proteins and collagen present in aortae, did not increase RNA yield. Subsequently, I found that TRIzol reagent in combination with the RNeasy Mini RNA extraction kit (Qiagen) was able to constantly provide satisfactory RNA yield from aortic samples. This combination also allowed convenient handling of the aqueous phase of TRIzol-

chloroform mixture in the RNA extraction protocol. RNeasy Mini columns bind and protect RNA from loss during propran-2-ol precipitation and resuspension steps, and the chances of introducing RNase contamination would therefore be less. However, the suggested on-column DNase digestion step was not performed because of concerns of lowering RNA yield. Nevertheless, results of the real-time quantitative PCR were not affected by genomic DNA contamination as the melting curve analysis of PCR products showed that only a single kind of amplicon was generated for each primer pair. As the primers were specifically designed to span introns, they should have generated amplicons of different sizes depending on whether the template was mRNA or genomic DNA.

The third issue in this set of experiments was the choice of reference gene. Housekeeping genes like *Gapdh* (coding for glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) and *Actb* (coding for β-actin) are genes commonly used as the internal reference; their expression is thought to be relatively constant in response to a whole host of cellular interventions. However, this notion is not necessarily true for septic stimuli as previous studies have shown that the expression of these two 'housekeeping' genes can be unstable (Hagihara *et al.*, 2004; Wang *et al.*, 2010). Hajihara *et al.* (2004) showed variable levels of *Gapdh* and *Actb* expression after stimulation of human hepatoblastoma and hepatoma cell line with pro-inflammatory cytokines. In contrast, several studies have shown that another reference gene, *Hmbs*, was more stably expressed during sepsis (Bilbault *et al.*, 2004; Alamdari *et al.*, 2008). While I found this also to be true in my hands, this may not always be

the case since expression of *Hmbs* is suppressed by hypoxia-inducible factor 1α (Vargas *et al.*, 2008). I did consider using 18S ribosomal RNA as the internal control in this study. Aside from the huge difference in amount between ribosomal and messenger RNA and the possible variation in their respective fractions between individual samples (Bustin and Nolan, 2004), it requires random hexamer for cDNA priming, which may lead to the synthesis of more than one copy of cDNA from a single mRNA template (Zhang and Byrne, 1999) and thus lead to nonspecific amplification.

3.3.1. Quantitative RT-PCR

At 6h, faecal peritonitis did not induce a significant increase in the vascular K_{ATP} subunit genes in aortae. On the contrary, SUR2B gene expression was suppressed (p <0.05; n = 5) in aortic smooth muscle. In mesenteric arteries at the same time point, there was an increase in K_{IR}6.1 mRNA, but this change did not reach statistical significance. However, at 24h after the onset of faecal peritonitis there was a 4-fold increase in K_{IR}6.1 gene expression, although SUR2B gene expression surprisingly remained unaltered (Figure 3.6). Thus the results in mesenteric arteries mirrored what I found in the primary cell culture model. At this stage, it is not clear why no such regulation was observed in aortae *in vivo*, but this will be further discussed later.

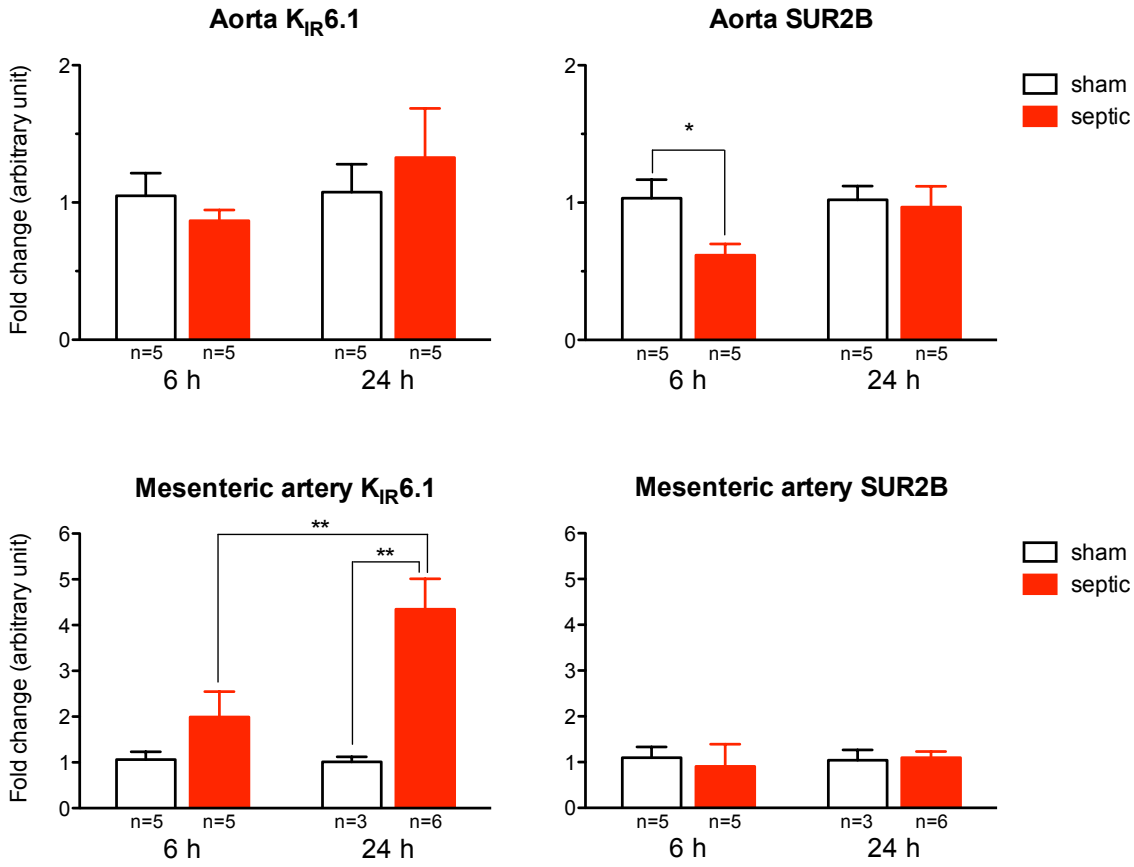


Figure 3.6. Real-time quantitative reverse transcription polymerase chain reaction of the vascular K_{ATP} channel subunit mRNA in the thoracic aorta and the mesenteric artery of rats subject to faecal peritonitis for 6 and 24h. *K_{ATP} channel subunit mRNA was normalized to that of the housekeeping gene,* Hmbs *(coding for hydroxymethylbilane synthase).* *p *<0.05,* **p *<0.01 (two-way ANOVA with Bonferroni* post-hoc *test).*

3.3.2. K_{ATP} channel functional activity

In my earlier experiments, LPS and IL-1β caused K_{ATP} channel hyperactivity in RASMC despite only $K_{IR}6.1$ subunit expressing being induced. Now that the same phenomenon occurred in *ex vivo* mesenteric arteries (but not in aortae) from septic rats, would these tissues also demonstrate K_{ATP} channel hyperactivity? In fact, if K_{ATP} channel basal hyperactivity could be detected in arterial tissues from septic animals, it would be the direct evidence that supports the channel's involvement in sepsis-induced vascular changes. Therefore K_{ATP} channel activity was measured in these tissues using $^{86}Rb^{+}$ efflux as a surrogate marker for K^{+} efflux (Bolton and Clapp, 1984). In each tissue, basal efflux of $^{86}Rb^{+}$ was measured for 15 min, then 10 μM of Lev was added to the assay buffer to see if it would stimulate efflux of the radioisotope. An increase in the response to a maximal dose of the K_{ATP} channel opener might reflect either an increase in the number of channels present in the plasma membrane or an increase in the open probability of the channel.

Figure 3.7 shows the results of the $^{86}Rb^{+}$ efflux experiment. Basal efflux from *ex vivo* vessels from septic rats was not increased at either 6h or 24h time point for the two blood vessel types. However, Lev-induced efflux was significantly increased at 24h in both aortae and mesenteric arteries, but not at 6h. In sham rats there was a trend towards a decrease in $^{86}Rb^{+}$ efflux evoked by Lev in aortae with time; no such trend was observed in mesenteric arteries.

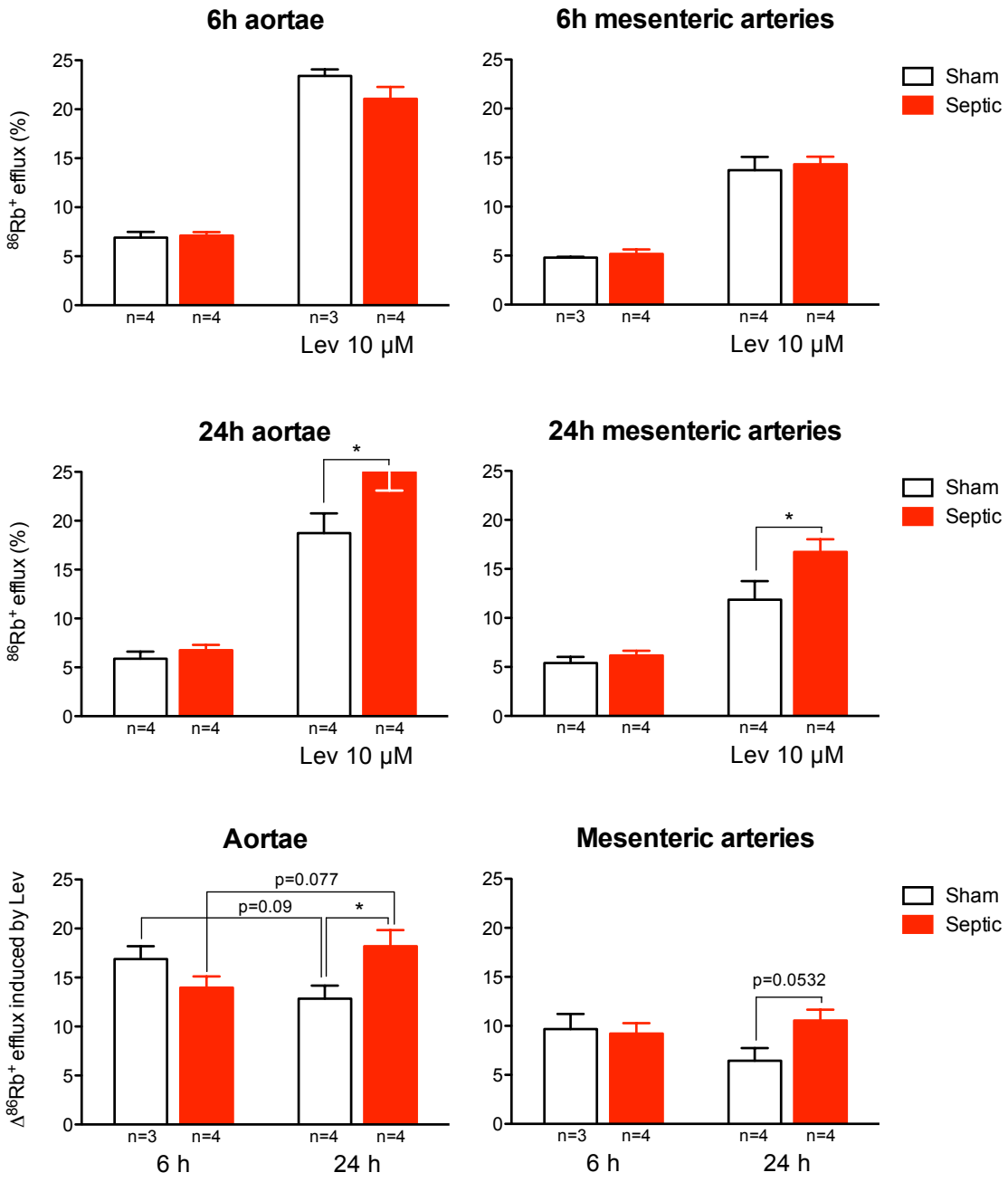


Figure 3.7. Basal and levcromakalim* (Lev)*-stimulated ^{86}rubidium ($^{86}Rb^+$) efflux (%) in aortae and mesenteric arteries taken from sham-operative control and septic rats subject to faecal peritonitis for 6 and 24h. Bars *represent percentage of efflux over 15 min normalized to total amount of $^{86}Rb^+$ in the blood vessel (Upper and middle panels). Change (Δ) in $^{86}Rb^+$ efflux (%) as induced by 10 µM Lev (Lower panel). *p <0.05 (two-way ANOVA with Bonferroni* post-hoc *test).*

3.4. Channel proteins

Questions arose from the results of the aforementioned experiments. The first concerns the non-paralleled changes in gene expression between the two channel subunits induced by septic stimuli, which occurred in both cultured primary cell and *ex vivo* tissue models. Thus whether an increase in gene expression of only the $K_{IR}6.1$ subunit can lead to channel hyperactivity is not clear. In other words, was channel hyperactivity observed in RASMC and mesenteric arteries actually originating from their increased subunit transcriptional expression? Channel gene transcription and translation do not necessarily lead to composition of functioning channels, and it may well be that the number of channels that are induced by sepsis does not correlate well with the level of transcriptional expression. For $K_{IR}6.2$/SUR1 K_{ATP} channels, the newly synthesized K_{IR} subunit protein rapidly gets degraded if not combined with the SUR subunit (Crane and Aguilar-Bryan, 2004). Thus an increase in $K_{IR}6.1$ transcriptional expression does not necessarily lead to a proportional increase in channel hyperactivity.

The second question arose from the observation that the stimulated hyperactivity of the channel in *ex vivo* aortae from 24h septic animals was not associated with an increase in channel gene expression. A single arterial smooth muscle cell has ~100-500 K_{ATP} channels, and normally very few of them remain in open state at a given time (Nelson and Quayle, 1995). Such a low "open-state probability" (P_o) is likely to result from inhibition of the channel by intracellular ATP, of which the physiological concentration is in the millimolar range (Kajioka *et al.*, 1991; Quayle *et al.*, 1994). Suppose 10 in 500 K_{ATP}

channels remain open at a given time as the basal state (P_o = 0.02). The opening of one additional channel is equivalent to the synthesis of additional 500. Why would the body choose to mobilize the whole channel-synthesizing mechanism to produce 500 new channels instead of opening just one amongst the 500 channels that are already there?

To answer these questions, it is important to determine the actual number of channels in plasma membrane, and the amount of channel proteins in a cell. Only then can channel gene expression and formation of functioning channels be correlated. I sought to measure the amount of subunit proteins in whole cell proteins as well as membrane fraction using Western blotting, hoping that I could demonstrate differences in protein production and plasmalemmal channel number.

Unfortunately, I encountered problems with the setup of Western blot experiment, and as a result this part of the study was unsuccessful. The first problem was separating membrane fraction of proteins. I employed the method devised by Kwan *et al.* (1984) to separate proteins extracted from aortic smooth muscle, but could not obtain an amount sufficient for Western blot. Then I went back to RASMC and started with crude cell homogenate, only to realize that a very high amount of whole-cell protein (50 µg per lane) is needed to show a visible SUR2B band on the blot. This suggested that using Western blot to detect channel subunit proteins in membrane fraction of proteins would be very difficult, given there would be substantially less protein in membrane fraction. Despite these difficulties, Zhou *et al.* (2007) have managed to generate blot for

SUR2B using 10 μg membrane protein using an anti-SUR2B antibody they generated themselves.

The second problem lies in the antibodies for K_{ATP} channel subunits used. The ability of the anti-SUR-2B (C-15; Santa Cruz) to bind specifically to SUR2B without cross-reaction with SUR2A remains doubtful given the close homology between these two splice variants. Moreover, the anti-K_{IR}6.1 (R-14; Santa Cruz) was shown to actually bind to two mitochondrial proteins (NADP-dehydrogenase flavoprotein 1 and isocitrate dehydrogenase) rather than K_{IR}6.1 (Foster *et al.*, 2008). Morrissey *et al.* (2005) also found that the same antibody failed to detect the subunit in Western blotting and immunohistochemistry, even though it seemed to work well in three other studies probing myocardium, aortae and cerebral arteries (Sampson *et al.*, 2004; Ploug *et al.*, 2006; Philip-Couderc *et al.*, 2008). Interestingly, none of these studies mentioned which kind of transfer solution they used (see below).

The third problem regarded the nature of channel subunits as membrane proteins, which often have anomalous migration during SDS-polyacrylamide gel electrophoresis (Rath *et al.*, 2009). This often raises confusion as to the location of the bands. Zhou *et al.* (2007) generated specific anti-SUR2B antibody that revealed bands at 110 kD, and Philip-Couderc *et al.* (2008) obtained a similar result. Figure 3.8 shows the result of my Western blotting experiment using RASMC whole-cell protein (50 μg per lane). The SUR2B bands were located just below 130 kD mark, rather than the predicted size of 174 kD, raising doubts as to whether this protein band is indeed SUR2B, unless it undergoes

degradation. The signal intensity of these SUR2B bands was not measured, for the experiment was still being optimized when I had to leave UCL.

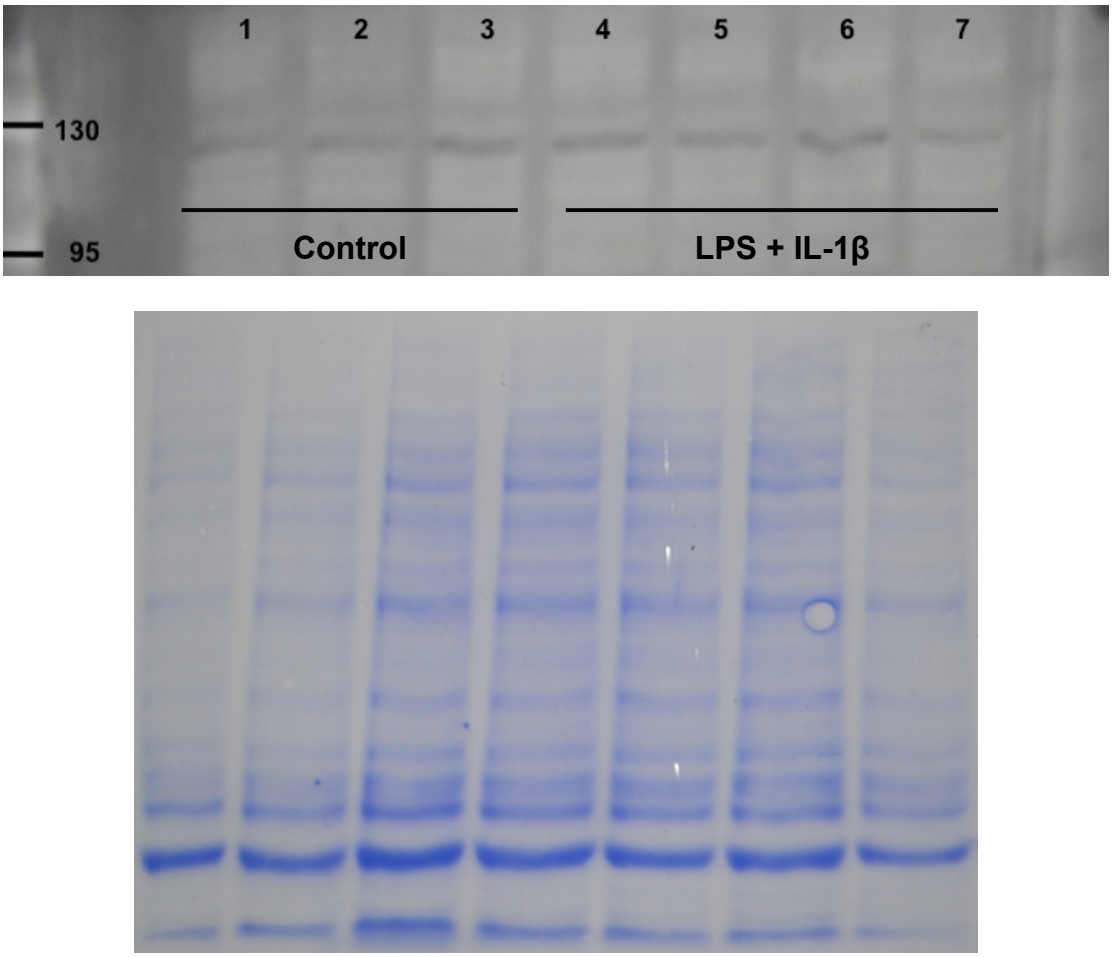


Figure 3.8. Western blot of whole-cell protein from quiescent RASMC subject to lipopolysaccharide (*LPS*; 1 μg·ml^{-1}) and interleukin (*IL*)-1β (10 ng·ml^{-1}) for 48h. a*) instead of the band appearing at the predicted 174-kD position, the bands were detected at ~110-kD. Fifty μg of protein were loaded. **b**) the blotted membrane was stained with Coomassie brilliant blue R for normalizing the amount of proteins loaded in each well.*

3.5. Discussion

The results of experiments presented in this chapter showed that (i) RASMC subject to septic stimuli or *ex vivo* mesenteric arteries from 24h septic rats had an increased gene expression of $K_{IR}6.1$ but not SUR2B subunit; (ii) the increased $K_{IR}6.1$ gene expression in RASMC was prevented by pre-treatment with the NOS2 inhibitor, 1400W, suggesting expression was linked to NOS2 induction; and (iii) while the increased $K_{IR}6.1$ gene expression in mesenteric arteries was associated with an increase in Lev-stimulated channel activity, the aortae from the same rats had an increased channel activity which, in contrast, was not associated with gene induction. This suggested that channel kinetics may also be altered during sepsis. This latter finding raised doubts as to the extent to which abnormal K_{ATP} channel gene expression can contribute to the sepsis-induced vascular changes.

The unparallel increase in vascular K_{ATP} subunit gene expression is not an uncommon finding. Rat embryonic heart-derived H9c2 cells subject to hypoxia (partial pressure 100 mmHg) or 17β-estradiol (100 nM) for 24h showed an increased SUR2A but not $K_{IR}6.2$ gene expression (Crawford *et al.*, 2003; Ren *et al.*, 2003). In contrast, an increase in $K_{IR}6.x$ but not SUR2 gene expression was observed in the setting of ischaemia and chronic inflammation (Akao *et al.*, 1997; Sgard *et al.*, 2000; Jin *et al.*, 2004). In the absence of SUR gene induction, the increased $K_{IR}6.x$ gene expression may still lead to an increased channel activity, depending on the amount of SUR2B reserve in the cell (van Bever *et al.*, 2004). Additionally synthesized $K_{IR}6.1$ may be degraded (Crane

and Aguilar-Bryan, 2004) or participate in other physiological processes that remain to be defined, *e.g.* the modulation of intracellular Ca^{2+} handling and muscle contraction (Ng *et al.*, 2010)

NO regulates gene expression mostly through indirect mechanisms such as modulating transcriptional factors (*e.g.*, NF-κB, AP-1), mRNA translation and stability, and processing of gene products (Bogdan, 2001a; Pfeilschifter *et al.*, 2001). Importantly, the proper functioning of the NF-κB signaling pathway seems to rely heavily on iNOS activity (Hierholzer *et al.*, 1998; van't Hof *et al.*, 2000). This may explain why 1400W prevented $K_{IR}6.1$ gene from being induced by LPS and IL-1β in RASMC, as Shi *et al.* (2010) reported that sepsis-induced K_{ATP} channel gene expression of $K_{IR}6.1$ and SUR2B was driven by NF-κB. This study also demonstrated that actinomycin was able to inhibit the production of channel mRNA. It would have been helpful if the K_{ATP} channel activity was measured in cells subject to LPS and actinomycin: the absence of an increased channel activity in these cells would indicate the role of channel gene induction in augmenting channel activity, whereas channel hyperactivity signifies altered channel kinetics during sepsis. The author did show a temporal increase in channel activity in parallel with gene expression, suggesting channel kinetics probably remained unaltered in this model.

An increase in basal $^{86}Rb^{+}$ efflux in arteries from septic animals did not appear as expected. This may be due to the limited sensitivity of the assay to pick up small increases in the opening of the vascular K_{ATP} channel. But how much of a change in $^{86}Rb^{+}$ efflux should be expected in this scenario? Suppose sepsis can

increase basal K_{ATP} channel activity by 50% (Shi *et al.*, 2010), and the change the $^{86}Rb^{+}$ efflux induced by 10 μM Lev represents a 5-fold increase in basal activity (Nelson and Quayle, 1995), it could be estimated that in the current experiment, the basal K_{ATP} channel activity is about 2.6% of $^{86}Rb^{+}$ efflux in aortae and 1.3% in mesenteric arteries, and sepsis should increase the efflux by 1.3% in aortae and 0.6% in mesenteric arteries, respectively. Considering the standard deviation of $^{86}Rb^{+}$ efflux measured in these vessels and the mortality of the current model (see **4.2**), about 75 24h septic rats need to be set up to achieve a statistic power of 70% for the difference in the mesenteric artery to be significant. For the difference in the aorta, 29 rats need to be set up. Thus the $^{86}Rb^{+}$ efflux experiment might be less ideal for demonstrating the change in basal K_{ATP} channel activity induced by sepsis. One unexpected finding was the decrease in Lev-stimulated $^{86}Rb^{+}$ efflux in aortae from sham rats at 24h, indicating the presence of higher channel activity at 6h which later recovered. Such stimulation may have originated from the trauma of instrumentation, but this needs to be confirmed by comparing to the results using aortae from the naive rats.

The major deficiencies for this part of the study are the inability to demonstrate an increase in channel protein production and surface expression in correspondence with channel gene induction. With suspicion to the specificity of the commercially available antibodies (Foster *et al.*, 2008), I did not, however, seek to generate the antibody by myself or to obtain it from other researchers. Electrophysiological experiments were attempted by an experienced and skillful postdoctoral fellow in the host lab so as to measure the plasmalemmal channel

number in freshly isolated smooth muscle cells from *ex vivo* rat aortae, but difficulties were encountered and unfortunately the experiment could not be set up in time.

In summary, $K_{IR}6.1$, the pore-forming subunit of vascular K_{ATP} channel, was inducible by septic stimuli, but the regulatory SUR2B subunit was not. In cultured RASMC, increased $K_{IR}6.1$ expression was associated with an increase in K_{ATP} channel function, and both phenomena were reversible by NOS2 inhibition. *Ex vivo* aortae and mesenteric arteries from rats subject to faecal peritonitis for 24h showed an increase in Lev-stimulated K_{ATP} channel activity, but only mesenteric arteries had increased $K_{IR}6.1$ expression. *Ex vivo* arteries had not an increase in basal K_{ATP} channel activity, possibly owing to the limited sensitivity of the $^{86}Rb^{+}$ efflux experiment to detect its change as induced by sepsis. The increase in channel number inducible by sepsis may be limited by the SUR2B reserve in arterial smooth muscle cell.

Chapter Four Characterization of the *in vivo* rat model of faecal peritonitis

4.1. Introduction

In the K_{ATP} subunit gene expression experiments I saw a marked increase in $K_{IR}6.1$ mRNA in rat aortic smooth muscle cell (RASMC) stimulated by lipopolysaccharide (LPS) and interleukin (IL)-1β for 48h, however I did not find this in *ex vivo* aortae taken from rats after 24h of faecal peritonitis. Although measurement at a later timepoint may have demonstrated induction of the $K_{IR}6.1$ subunit in aortae, the rats were unlikely to extend much beyond 24h. The severity of this model had clearly changed since its original description (Brealey *et al.*, 2004) where a 72h mortality of 40% was reported. Because of potential concerns of fluid overload, a revised model only used half the volume of fluid resuscitation (Barrett *et al.*, 2007a) and achieved mortality rates of 32% at 24h, and 64% at 48h. In both these studies, haemodynamics, renal and liver function, and mortality were all assessed. I used the modified (lower) fluid regimen and further assessed the degree of tissue hypoperfusion and the levels of NO generated, as well as the animals' pressor response to vasoactive therapy. The higher mortality I witnessed was common to other investigators within the lab at the time and suggests an altered phenotype of the rats in response to the fixed septic insult. Meanwhile, as part of another study running concurrently within the lab, rats were receiving a faecal peritonitis insult but

received no fluid resuscitation. I have collated data from these different models to assist with characterization. I gratefully acknowledge the assistance of Drs. Alex Dyson (arterial base excess and lactate levels in non-reserpinized sham and septic rats at 24h) and Zhen Wang (survival time and blood pressures of faecal peritonitis rats without fluid resuscitation).

4.2. Faecal peritonitis model is a severe model of septic shock

Using an identical design to the Barrett model with the modified fluid regimen, I found a 24h mortality of 48.2%. In the original study (Brealey *et al.*, 2004), a scoring system was designed to grade disease severity according to the clinical presentations of these animals (Table 4.1). Results of the grading system correlated well with changes in MAP in septic rats at 24h, and the degree of

Table 4.1. Severity scoring system of rats subject to faecal peritonitis. *Table reproduced from Brealey et al. (2004).*

	Mild	Moderate	Severe
Appearance	Hunched Piloerection No bloating	Hunched Marked piloerection Bloated abdomen	Marked piloerection Markedly bloated abdomen Conjunctival injection
Alertness	Alert Occasional interest in environment Moves freely	Depressed level of alertness Little interest in environment Moves with difficulty	Markedly (or absent) depressed level of alertness No interest in environment No movement
MAP	>90 mmHg	75–90 mmHg	<75 mmHg

kidney and liver dysfunction. However, surviving septic rats presented with different disease severity at 24h and 48h. Therefore, septic animals categorized to the same disease severity at different timepoints may actually represent distinct disease processes.

In my project, 164 rats (mean weight, 308 ± 3 g) were successfully instrumented and made uneventful post-operative recoveries. Of the 148 animals, 68 (54 for 6h; 15 for 24h) were used as sham-operative controls, while peritonitis was induced in 87 (66 for 6h; 29 for 24h). Control animals appeared outwardly normal throughout. The septic animals showed features of illness, including hunched posture, piloerection, and decreased movement and alertness from 12h after faecal slurry injection. Mortality rates for septic rats at 6 and 24h was 6.1% and 48.2%, respectively, and 0% in controls. Despite 24h septic rats had a high mortality rate that might be related to the excessive amount of faecal slurry injected, I did not alter the faecal slurry dose so as to ensure the targeted disease severity could be obtained at 6h.

4.3. Severity of tissue hypoperfusion and levels of NO generation

The levels of arterial base excess (negative values indicate a deficit) and plasma lactate were used as surrogate markers of tissue hypoperfusion. In the septic animals a metabolic acidosis was present as early as 6h while plasma lactate levels were elevated after 24h. Plasma nitrate/nitrite (NOx) levels were elevated at 6h and, in surviving septic animals, the levels remained high (Figure 4.1).

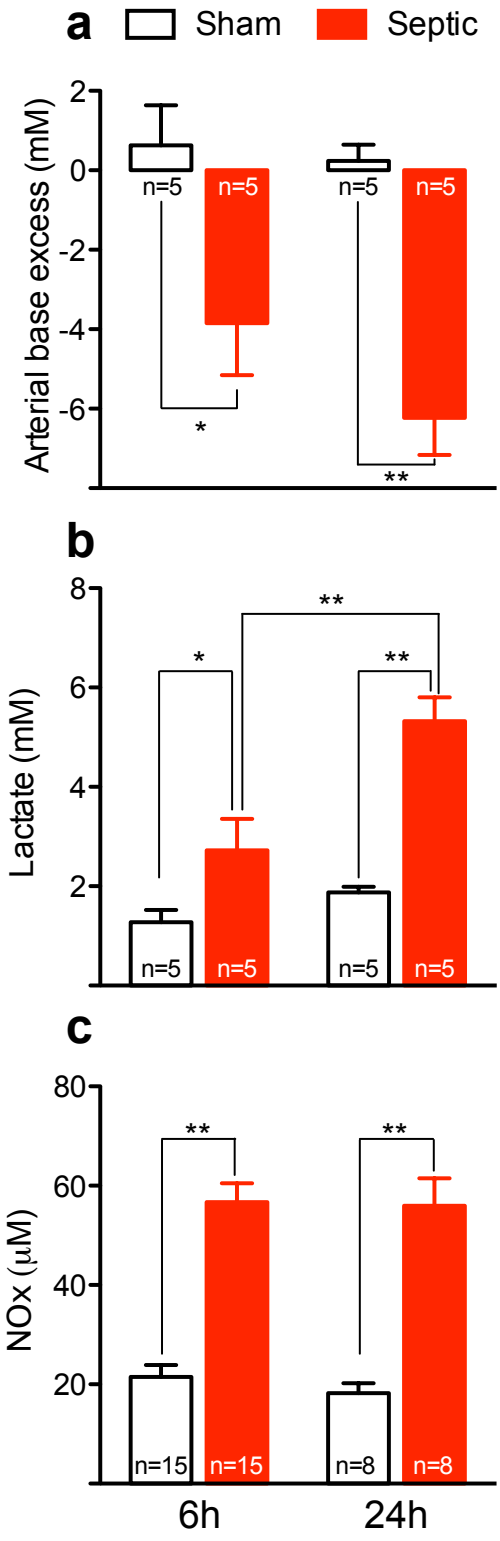


Figure 4.1. Arterial base excess (a), plasma lactate (b) and nitrate/nitrite (NOx; c) levels of rats subject to faecal peritonitis for 6 and 24h. *A negative value of base excess indicates a deficit.* *p *<0.05,* **p *<0.01 (two-way ANOVA with Bonferroni* post-hoc *test).*

4.4. Effect of fluid resuscitation

An important feature of this model is the incorporation of intravenous fluid resuscitation. As sepsis can induce changes leading to reductions in effective circulatory volume, its repletion is essential in maintaining haemodynamic stability. Moreover, the hyperdynamic state that characterizes septic shock can be present only after volume repletion (Carroll and Snyder, 1982; Otero *et al.*, 2006). In clinical practice, fluid resuscitation is prioritized as the first line treatment for septic patients with haemodynamic instability (Hollenberg *et al.*, 2004).

The importance of fluid resuscitation can be further demonstrated by the finding that none of the 24 rats subjected to faecal peritonitis who were not fluid resuscitated survived beyond 24h. Mean surviving time was 13.3 ± 1.0h, and eleven (46%) died within 10h. Five hours after intraperitoneal injection of faecal slurry, these rats developed a more severe metabolic acidosis (arterial base excess -10.4 ± 2.9 mM, n = 7) than their fluid-resuscitated counterparts (p = 0.016). Of note, none of the 24 rats developed hypotension at 5h and three quarters manifested no hypotension (defined as drops in mean arterial pressure [MAP] of >20%) until the last hour before death. This likely reflects the ongoing ability of these awake animals to mount a vasopressor response, despite the decreased vascular reactivity to exogenous catecholamines.

A reasonable question of the model to address is whether the volume of fluid administered (10 $ml \cdot kg^{-1} \cdot h^{-1}$ for 22 h) was adequate, insufficient or even excessive. Indeed, excessive fluid can also lead to adverse outcomes (Brandt

et al., 2009). At laparotomy, all the septic rats showed evidence of peritoneal inflammation that varied from small-volume ascites and mild bowel oedema to gross bowel distension with purulent ascites and multiple adhesions. In contrast, none of the sham rats had ascites. Importantly, many septic rats developed large-volume ascites that amounted up to 8 ml at 6h. This could have counterbalanced a large part of the fluid administered. Concurrent echocardiographic studies in the lab using this rat model by Dr. Alain Rudiger revealed this fluid regimen to be acceptable though severity, as reflected by the degree of ascites, clearly varied markedly between animals. For simplicity and constancy of approach, I gave a fixed fluid regimen to all animals and no individual titration depended on the degree of sepsis-induced severity. Whether this may have contributed to any confounding of my results requires further study.

4.5. Baseline blood pressure and response to norepinephrine

The septic rats had an elevated MAP at 6h post-slurry whereas this had fallen by 24h (Figure 4.2**b**). This increase in MAP at 6h is likely to have resulted from increased sympathetic tone as septic rats developed tachycardia, with their heart rates increasing from 366 ± 12 beats per min (bpm) at 0h, to 519 ± 14 bpm at 6h (n = 8, p <0.01). At 24h, the septic rats remained tachycardic (520 ± 34 bpm; n = 4). By comparison, the heart rates of sham rats at 0 and 6h were 329 ± 14 and 344 ± 9 bpm, respectively (n = 6, p = NS). Irrespective of their blood pressure, septic rats had an attenuated pressor response to NE at both

6h and 24h timepoints (Figure 4.3). Thus, vascular hyporeactivity preceded hypotension and was already present at 6h.

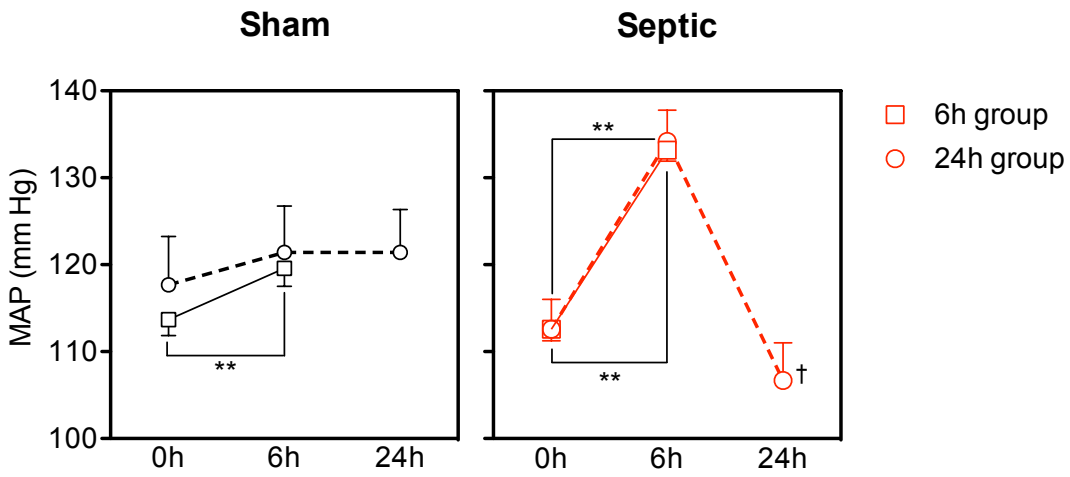


Figure 4.2. Baseline mean arterial pressure (MAP) at 0, 6 and 24h in sham (n = 50 in 6h group and 11 in 24h group) and septic (n = 57 in 6h group and 19 in 24h group) rats with fecal peritonitis. *Dotted lines represent the 24h group.* **p *<0.01;* †p *<0.05 compared to sham rats at the same timepoint (two-way ANOVA repeated measures with Bonferroni* post-hoc *test).*

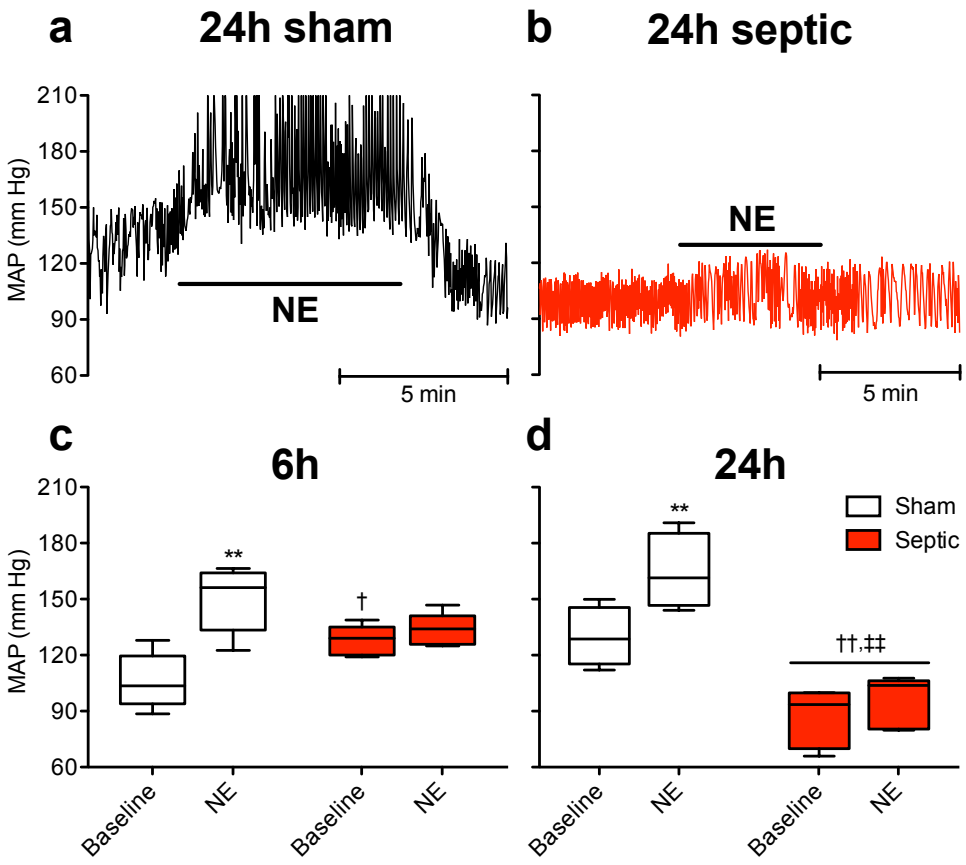


Figure 4.3. Representative blood pressure (*MAP*) traces of (a) sham-operated and (b) septic rats pre- and post-administration of norepinephrine (*NE*; 0.5 µg·kg^{-1}·min^{-1} i.v.) at the 24h timepoint. The pressor effect is shown in sham and septic rats at (c) 6h and (d) 24h. Boxes *represent median and interquartile range (*n = 5*).* **p *<0.01 compared to baseline pressure;* †p *<0.05,* ††p *<0.01 compared to sham animals;* ‡‡p *<0.01 compared to 6h (two-way ANOVA repeated measures with Bonferroni* post-hoc *test).*

4.6. Effects of NOS inhibition

Since excessive generation of NO is a major factor that contributes to sepsis-induced vascular changes, I assessed whether these could be reversed by inhibiting NO production. I chose to use SEITU as it not only inhibits NOS2 (inducible) but also inhibits NOS3 (endothelial) (Boer *et al.*, 2000). The latter isoform may be activated in early sepsis and also contribute to vascular hyporeactivity (Thiemermann, 1997). Given septic rats had a higher level of NO production (see Figure 4.1), I expected SEITU should have had a stronger pressor effect in these animals. However, my findings did not confirm this

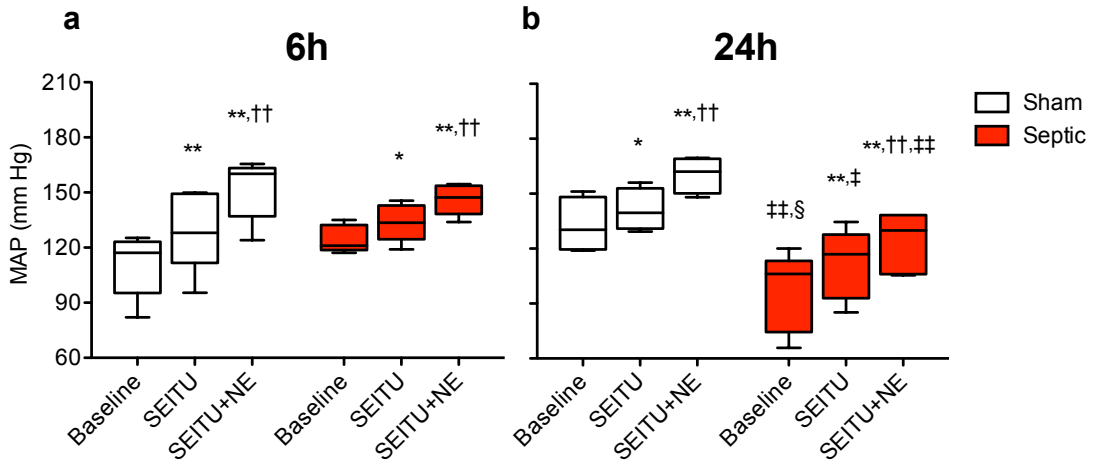


Figure 4.4. Mean arterial pressures (*MAP*) of nonreserpinized septic rats before and after administration of* S-*ethylisothiourea (*SEITU*; 0.1 mg·kg^{-1}·min^{-1}* i.v.*), with and without norepinephrine (*NE*; 0.5 µg·kg^{-1}·min^{-1}* i.v.*) at 6 (a) and 24h (b). Boxes *represent median and interquartile range (*n = 5*).* *p *<0.05,* **p *<0.01 compared to baseline pressure;* ††p *<0.01 compared to pressure after SEITU administration;* ‡p *<0.05,* ‡p *<0.01 compared to sham animals at the same timepoint;* §p *<0.05 compared to pressure at 6h timepoint (two-way ANOVA repeated measures with Bonferroni* post-hoc *test).*

(Figure 4.4). At 6h, when blood pressures were being maintained in the septic animals, the pressor response to SEITU was similar in both sham and septic rats but the subsequent administration of NE still produced a significantly greater response in the sham animals. At 24h, however, when blood pressures in the septic animals were lower, there was a significant rise in BP with SEITU over and above that seen in the sham animals. However, the subsequent response to NE was blunted in both sham and septic animals (Figure 4.4**b**).

4.7. Discussion

The data presented in this chapter demonstrated that at 6h post-faecal slurry injection septic rats had raised blood pressure but an attenuated pressor response to NE. The pressor response of 6h septic rats to SEITU was less prominent, contradicting with their augmented NO synthesis *in vivo*. At 24h post-faecal slurry injection septic rats developed hypotension and vascular hyporeactivity to NE. Septic rats with faecal peritonitis could maintain their blood pressure till moribund, even in the absence of intravenous fluid resuscitation. This could mean that the blood pressure-maintaining mechanisms had a high feedback gain (Figure 1.1), unlike septic patients who often develop hypotension because the body cannot fully compensate the vascular changes. However, this could also mean that septic rats were less tolerable to hypotension and fell dead soon after its development. Either way, in this model the blood pressure may not correlate well with disease severity, an observation that was seldom recognized before.

The concurrent presence of hypertension and vascular hyporeactivity to NE in the 6h septic rat was particularly confusing as to whether this model actually reproduces sepsis-induced vascular changes. The fact that the rats in the current model were not anaesthetized may explain the disparity, as anaesthesia may pose inhibitory effect on the sympathetic efferent nerve activity (Seagard *et al.*, 1984) and the baroreceptor reflex (Saeki *et al.*, 1996). How would the septic rat's pressor response to NE and SEITU be affected by a 'more intact' state of compensatory mechanisms? One scenario that I speculate might be that if the septic rat had already relied on reflex sympathetic stimulation to maintain blood pressure, the *i.v.* infusion of NE or/and SEITU would have partially relieved the reflex stimulation, resulting in a decrease in background sympathetic tone while the animal's blood pressure remained the same, giving rise to an 'attenuated' pressor effect of these agents. The sympathetic nerve activity of the 6h septic rats before and after administration of NE and SEITU should be measured so as to understand whether the background sympathetic tone is decreased. If yes, then the model may risk underestimating the pressor effect of a vasoactive agent. To my knowledge, this observation of the concurrent presence of hypertension and vascular hyporeactivity in septic rats has never been described before.

In this model, the host lab previously showed that the 24h plasma level of NE was four-fold higher in septic rats compared to sham-operated controls (Barrett *et al.*, 2007a). The increased sympathetic tone may be related to a maintained response to ongoing inflammation system (Zhou *et al.*, 1991; Hahn *et al.*, 1995) plus also any associated discomfort (the animals did not appear to be in

obvious pain) and/or any uncorrected hypovolaemia. Moreover, sepsis may pose a direct effect on sympathetic tone. Endotoxin can stimulate sympathetic nerves directly in rats (Zhou *et al.*, 1991) though in healthy humans it may conversely inhibit them (Sayk *et al.*, 2008). Pancoto *et al.* (2008) reported an increase in autonomic centre neuronal apoptosis in some patients dying from septic shock. The ability of the autonomic nervous system to modulate tone in the heart and blood vessels may also become impaired (Annane *et al.*, 1999).

Sepsis initially stimulates NOS3 activity and later induces expression of NOS2, both of which contribute to the excessive amounts of NO that induces vasorelaxation and vasoplegia (Thiemermann, 1997). The results seen with this current model did reveal a stronger pressor response to the non-selective NOS inhibitor, SEITU in septic rats at 24h; however, NOS inhibition failed to normalize the blood pressure in these animals. This suggests the presence of mechanisms other than NO that contribute to hypotension. Potential other mediators include prostacyclin (Zardi *et al.*, 2007), calcitonin gene-related peptide (Joyce *et al.*, 1990; Tang *et al.*, 1997), and increase in Na^+-K^+ pump activity (Chen *et al.*, 2003). The accentuated pressor response to SEITU seen in sham rats at 6h may have resulted from anaesthesia and the instrumentation procedure that stimulated NOS activity in these animals. The dose of SEITU used already lowered cardiac output (Dyson A, unpublished observations), and rats seemed intolerable to higher doses of this agent (data not shown). The decrease in cardiac output may be the result of an increased cardiac afterload, a decreased coronary blood flow, or/and a decrease in reflex sympathetic stimulation as was discussed earlier. This decrease in cardiac output may

underlie the attenuated pressor response to SEITU in septic rats. Likewise, SEITU did not fully reverse the attenuated pressor response to NE in septic rats, either at the 6h or 24h timepoints. The vasoplegic effect may originate from NO stored *in vivo* as *S*-nitrosothiol and nitrite (Rodriguez *et al.*, 2003; Dejam *et al.*, 2005). Consistent with this notion, (da Silva-Santos *et al.*, 2002) showed that rats exposed to LPS for 24h had an attenuated pressor response to phenylephrine, which could not be reversed by L-NAME, but could be reversed by ODQ. They suggested that sGC activity contributed to the vasoplegia, and that this was not stimulated by newly synthesized NO.

In summary, the faecal peritonitis model used in this study was a severe model of sepsis. How this affects vascular reactivity requires further investigation. The septic animals were clinically unwell and developed signs of organ dysfunction yet I did not witness the expected greater effect from NOS inhibition, despite evidence of increased NO production. The relative maintenance in blood pressure occurred despite marked vascular hyporeactivity to NE. This finding implies that the rat's blood pressure-maintaining mechanisms has a high feedback gain.

Chapter Five Changes in blood pressure induced by K_{ATP} channel modulation

5.1. Introduction

As septic shock is characterized by arterial and venous vasodilatation, and the vascular K_{ATP} channel is an important regulator of vascular tone, it is reasonable to assume that vascular K_{ATP} channels may be stimulated during sepsis to induce arterial vasodilatation. Pharmacological agents that cause K_{ATP} channel opening can indeed induce hypotension that could be reversed by the K_{ATP} channel inhibitor glibenclamide (Singer *et al.*, 2005). However, the same drug was not able to reduce the dose of NE or shorten the duration of its usage in septic shock patients (Warrillow *et al.*, 2006). This may be related to an insufficient dose to block the ion-conducting subunit of the K_{ATP} channel, which requires a higher concentration than is likely ever to be given to patients (Buckley *et al.*, 2006). PNU-37883A, a vascular K_{ATP} channel inhibitor acting primarily on the pore-forming $K_{IR}6.1$ subunit, was able to reverse LPS-induced vascular hyporeactivity to NE in *ex vivo* rat mesenteric arteries, while glibenclamide was not (O'Brien *et al.*, 2005). However, when PNU-37883A was administered to anaesthetized rats with endotoxic shock, the pressor response in septic was not as marked as in sham rats (O'Brien *et al.*, 2009), possibly suggesting that sepsis may alter the channel in such a way as to make channel inhibitors less able to bind, irrespective of whether they bind to SUR or $K_{IR}6.x$ subunits (Wilson and Clapp, 2002). The importance of the vascular K_{ATP}

channels in causing vasodilatation and vascular hyporeactivity in sepsis thus remains difficult to tease out. The results from Kane *et al.* (2006) would support the notion that these channels do indeed contribute to the cardiovascular collapse in sepsis. They showed in a short term model of endotoxic shock that $K_{IR}6.1$ knockout (KO) mice did not become hypotensive in the presence of LPS, whereas they did in wild-type mice. However, surprisingly, endotoxic KO mice all died within 24h, whereas mortality in wild-type mice could be improved by the administration of the K_{ATP} channel opener pinacidil. They claimed that coronary vasospasm was the cause of death in the endotoxic $K_{IR}6.1$-KO mice due to the lack of functioning K_{ATP} channels in the coronary vascular bed (Miki *et al.*, 2002). Verapamil, a L-type Ca^{2+} channel blocker, dilated their coronary arteries and rescued some animals from dying. These results suggest that the vascular K_{ATP} channel participates in the modulation of vascular tone in health as well as disease states. At least in mice, the strategy of inhibiting vascular K_{ATP} channels to reverse septic shock is questionable, as it may do more harm than good.

After seeing the results of the *in vivo* pharmacology experiments involving PNU-37883A in anaesthetized rats (O'Brien *et al.*, 2009), I wondered whether their anaesthetized state, or the use of the anaesthetic agent isoflurane, which has K_{ATP} channel opening effects of its own (Iida *et al.*, 1998; Tanaka *et al.*, 2007), could have confounded the interpretation of results. I thus decided to test the pressor effect of PNU-37883A in conscious rats. Instead of the endotoxaemic model I used the faecal peritonitis model, thinking the latter more resembled real disease conditions, and presumably the resultant systemic inflammation

would be similar. Admittedly this replacement of the model did not entirely follow the scientific principles. Nevertheless, sham-operated rats still presented with a pressor response to PNU-37883A more prominent than septic rats irrespective of anaesthesia and the means to induce sepsis.

5.2. Effects of K_{ATP} channel modulation

PNU-37883A elevated mean arterial pressure (MAP) by approximately 25 mmHg in awake sham rats at both 6h and 24h timepoints (Figure 5.1**a, c, d**). This rise is comparable to that seen with PNU-37883A in anaesthetized rats (O'Brien *et al.*, 2009) and in K_{IR}6.1-KO compared with wild type mice (Kane *et al.*, 2006). As with NOS inhibition, I speculated that PNU-37883A should have a more potent pressor effect in septic rats who would supposedly have higher levels of vascular K_{ATP} channel activation. As with the findings in anaesthetized rats, PNU-37883A (at the dose used) had a less prominent pressor effect at both 6h and 24h in the septic animals compared to the sham-operated controls. Changes in MAP induced by PNU-37883A in septic rats at 6h and 24h were not significantly different (n = 5, p = NS), being 7.6 ± 1.4 mmHg and 11.2 ± 1.8 mmHg, respectively. As with the NOS inhibitor SEITU, PNU-37883A also failed to reverse the attenuated pressor response of NE in the septic animals (Figure 5.1).

As this could potentially be related to an insufficient dose of PNU-37883A to reverse the hyperactivity of the channel, I then constructed dose-response curves of PNU-37883A in sham and septic rats at the 6h timepoint. I found that

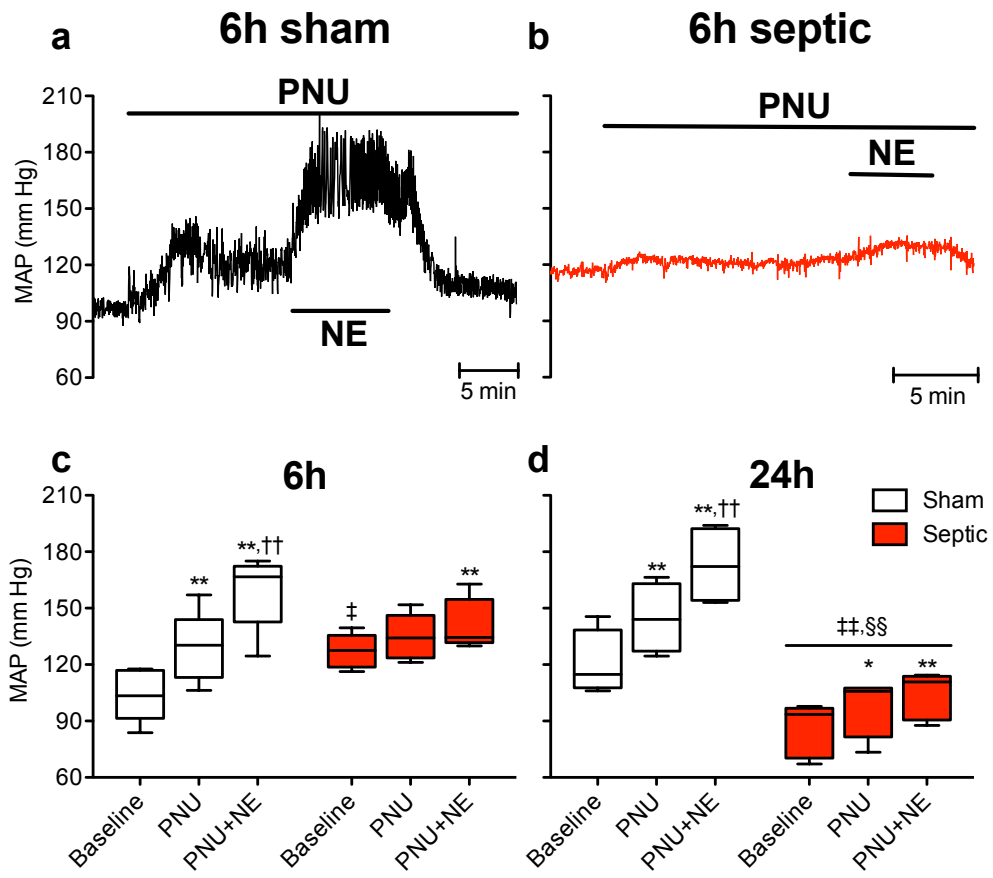


Figure 5.1. Representative blood pressure (*MAP*) traces from (a) sham-operated and (b) septic rats pre- and post-administration of PNU-37883A (*PNU*; 1.5 mg·kg^{-1} and 1.0 mg·kg^{-1}·h^{-1} i.v.) and norepinephrine (*NE*; 0.5 µg·kg^{-1}·min^{-1} i.v.), at the 6h timepoint. Subgroup data are shown pre- and post-administration of PNU-37883A ± NE at (c) 6h and (d) 24h. Boxes *represent median and interquartile range (*n = 5*).* *p *<0.05,* **p *<0.01 compared to baseline MAP;* ††p *<0.01 compared to MAP post-PNU administration;* ‡p *<0.05,* ‡‡p *<0.01 compared to sham-operated animals at the same timepoint;* §§p *<0.01 compared to MAP at 6h (two-way ANOVA repeated measures with Bonferroni* post-hoc *test).*

the dose used in the current study was already near the plateau of its maximal effect (Figure 5.2). Interestingly, the EC_{50} of PNU-37883A in sham and septic rats was not significantly different ($-\log EC_{50}$ = 0.51 ± 0.34 for sham, and 0.61 ± 0.57 for septic; *n* = 4).

As reasoned in **3.2.4** and **3.3.2**, septic rats should have an accentuated effect to levcromakalim (Lev) if either K_{ATP} channel genes were induced, leading to more channels in the plasma membrane, or if channel opening was potentiated by interaction with channel modulators (Kessler *et al.*, 1997). In both sham and septic animals, Lev increased vascular K_{ATP} channel opening, thereby relaxing arteries and lowering the blood pressure. As predicted, septic rats showed a bigger fall in MAP to Lev at 6h (Figure 5.3**c** and Figure 5.4). Such potentiation was not seen at the 24h timepoint where the same drop in MAP occurred from a lower baseline (Figure 5.3**b** and **d**). Of note, the EC_{50} of Lev in sham and septic rats at 6 and 24h were not significantly different. The $\log EC_{50}$ in sham and septic rats were 2.1 ± 2.8 and 1.3 ± 0.2 at 6h (*n* = 3), and 2.5 ± 5.5 and 1.7 ± 0.2 at 24h (*n* = 4) respectively.

My underlying assumption was that sepsis had induced a profound hyperactivity of the vascular K_{ATP} channel that could not be reversed with PNU-37883A, so administration of Lev would also be impossible to reverse by the same dose of PNU-37883A. Thus, I tested the efficacy of PNU-37883A to inhibit Lev-induced vasorelaxation at the same dose used in Figure 5.1. I was proven wrong again as this agent could successfully reverse Lev-induced hypotension in septic rats (Figure 5.4).

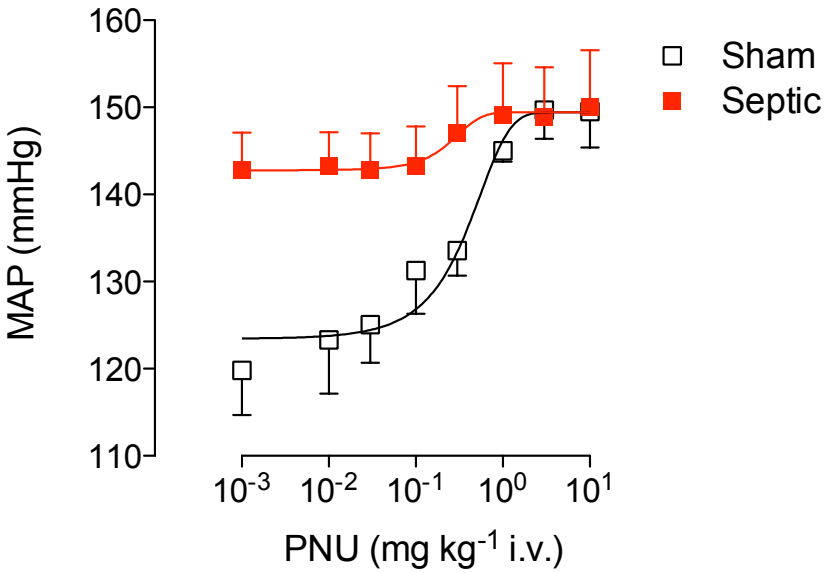


Figure 5.2. Dose-response curves of the pressor response of sham-operated (*sham*) and faecal peritonitis (*septic*) rats to PNU-37883A (*PNU*), a vascular K_{ATP} channel inhibitor acting on K_{IR}6.1 subunit. *Rats were subject to faecal peritonitis for 6h; all doses of PNU-37883A were given as 5-min infusions.* n = *4.* MAP = *mean arterial pressure.*

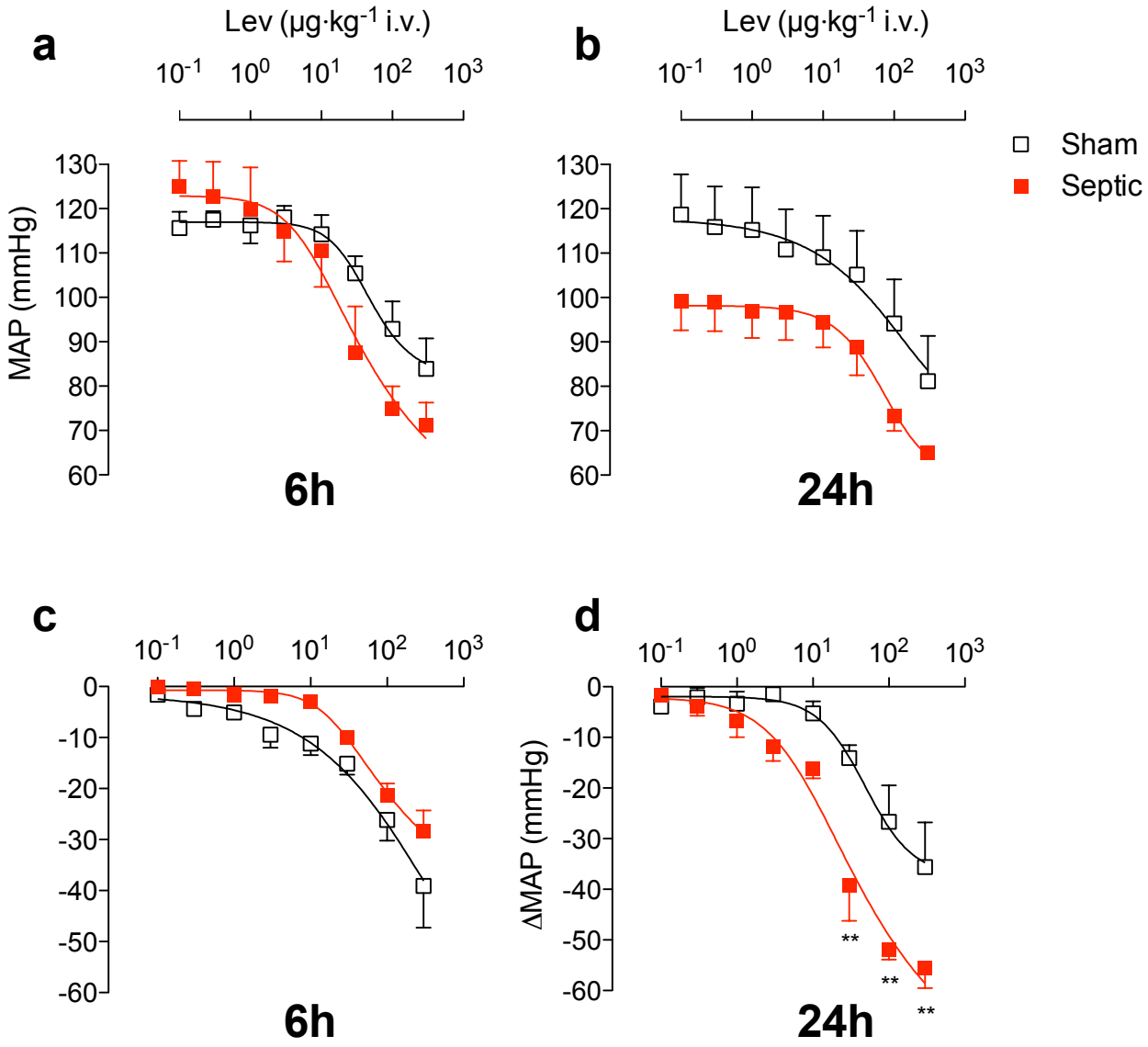


Figure 5.3. Dose-response curves assessing the effect of the vascular K_{ATP} channel opener levcromakalim (Lev) on mean arterial pressure (MAP, a and b) and its changes (ΔMAP, c and d) in sham-operated and septic rats at 6h (a and c; n = 3) and 24h (b and d; n = 4). ***p <0.01 compared to sham-operated controls (two-way ANOVA repeated measures with Bonferroni post-hoc test).*

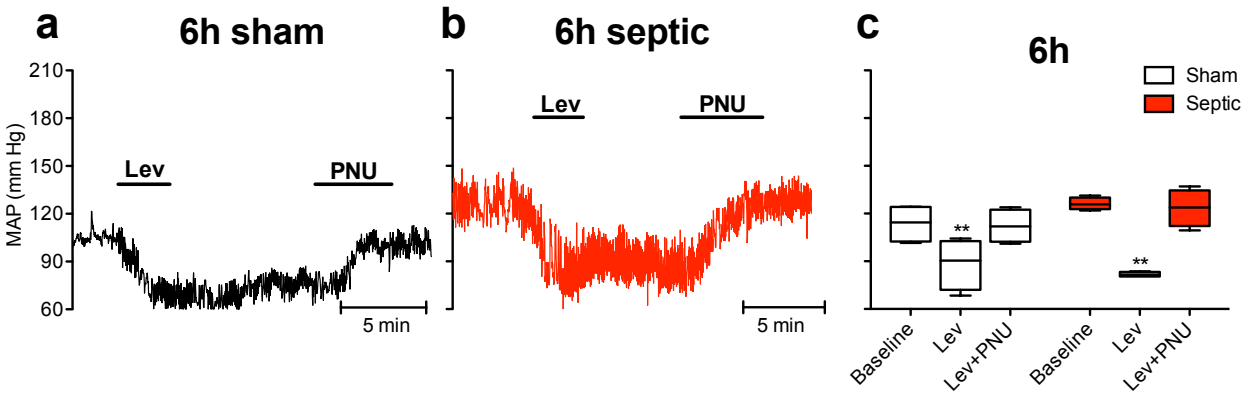


Figure 5.4. Representative blood pressure (*MAP*) traces from (a) sham-operated and (b) septic rats pre- and post-administration of levcromakalim (*Lev*; 150 µg·kg^{-1} i.v.) and PNU-37883A (*PNU*; 1.5 mg·kg^{-1} and 1.0 mg·kg^{-1}·h^{-1} i.v.), at the 6h timepoint. Subgroup data are shown pre- and post-administration of Lev ± PNU-37883A at (c) 6h. Boxes *represent median and interquartile range (*n = *4).* **p <*0.01 compared to baseline pressure (two-way ANOVA repeated measures with Bonferroni* post-hoc *test).*

Since septic rats presented with an elevated serum NOx level indicating excessive *in vivo* NO production, I wondered if this would cause the attenuation of the pressor response to PNU-37883A in these animals. The activity of the vascular K_{ATP} channel may be stimulated by NO, thus the inhibition of NOS might reduce this stimulation, resulting in a lower basal activity of the channel and an attenuated pressor response to PNU-37883A. In contrast, excessive NO may potentially make any increase in $[Ca^{2+}]_i$ induced by vascular K_{ATP} channel inhibition insufficient to reverse the vascular change. In this case the inhibition of NOS activity may conversely increase the pressor effect of PNU-37883A. Which effect would predominate remains uncertain.

Therefore at the 6h timepoint, I administered PNU-37883A to sham and septic rats on top of the continuous infusion of *S*-ethylisothiourea (SEITU). The resulting lesser increase in MAP in sham rats with PNU-37883A (Figure 5.5) suggests that vascular K_{ATP} channel activity may be stimulated by NOS3 activity in normal physiology. However, in septic rats the combination of SEITU and PNU-37883A infusion produced a greater pressor response than either agent alone, suggesting an additive effect of the two agents in our septic model, and the pressor effect of PNU-37883A seems less dependent on NOS activity.

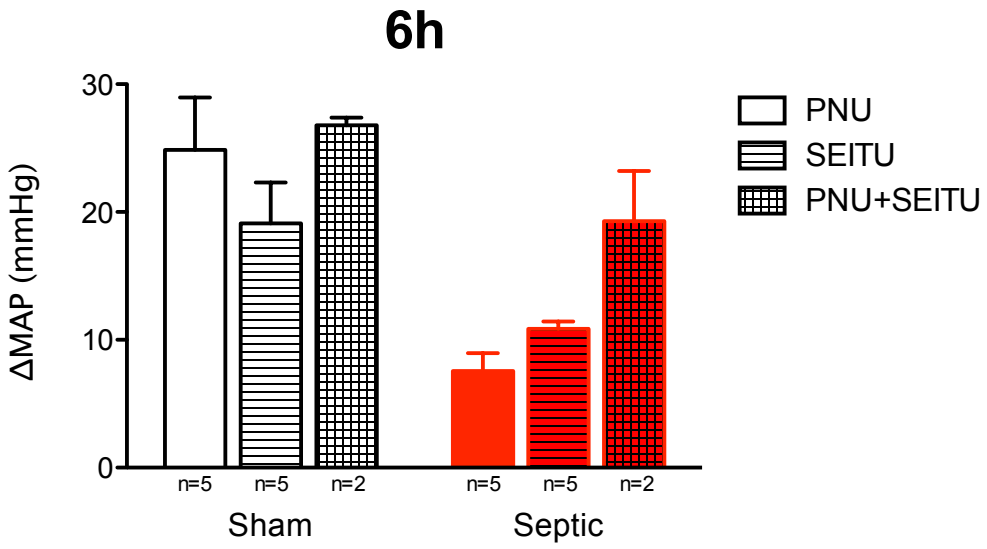


Figure 5.5. Changes in mean arterial pressure (*MAP*) in 6h sham-operated and septic rats with faecal peritonitis at 6h as induced by PNU-37883A (*PNU*; 1.5 mg·kg^{-1} and 1.0 mg·kg^{-1}·h^{-1} i.v.), S-ethylisothiourea (*SEITU*; 0.1 mg·kg^{-1}·min^{-1} i.v.), and co-administration of both drugs. *Changes in MAP induced by PNU or SEITU alone were the same results shown in* ***Figure 5.1c*** *and* ***Figure 4.4****, respectively.*

5.3. Modulation of compensatory mechanisms

It thus appeared that the dose of PNU-37883A was high enough to successfully reverse vasodilatation induced by a near-maximal dose of Lev, but in septic rats at either 6h or 24h timepoints its pressor effect was attenuated, and the K_{ATP} channel inhibitor could not reverse the vascular hyporeactivity to NE. The most straightforward conclusion is that the vascular K_{ATP} channel is not involved in the pathogenesis of septic shock in this model. Another possibility is that the mechanism induced by sepsis to open the channel could not be inhibited either by PNU-37883A or glibenclamide, *i.e.* sepsis may alter the pharmacological

behaviour of the vascular K_{ATP} channel. The third possibility is that the anaesthetics used in previous sepsis studies are major contributors to K_{ATP} channel activation as most anaesthetic agents are recognized modulators of vascular K_{ATP} channel activity (Kato *et al.*, 2000; Kawano *et al.*, 2004, 2005; Nakamura *et al.*, 2007). To circumvent this, I explored the use of other anaesthetic agents that do not activate the channel to see whether a pressor effect of PNU-37883A could then be observed in septic animals. I decided to use midazolam as this has previously been reported to not alter vascular K_{ATP} channel activity *in vitro* (Nakamura *et al.*, 2007). While midazolam is not actually an anaesthetic agent, the dose I selected has been reported to suppress the somatosympathetic reflex discharges in cats (Iida *et al.*, 2007). Higher doses of midazolam was avoided to prevent vasodilatation (Shiraishi *et al.*, 1997).

Except for a near-significant accentuation of the pressor response to PNU-37883A in sham rats, midazolam had no other effect on pressor responses (Figure 5.6). However, the results seemed to imply a potential effect of the underlying sympathetic tone on the vascular K_{ATP} channel activity: midazolam might have suppressed the sympathetic tone of sham rats and somehow rendered them 'hyperactive' to PNU-37883A; in contrast, septic rats with high sympathetic tone had an attenuated pressor response to this agent. As vascular K_{ATP} channels can be inhibited by phenylephrine (Bonev and Nelson, 1996), it is possible that high sympathetic tone also inhibits the channel *in vivo*, thus rendering septic rats unresponsive to PNU-37883A. I thus decided to remove this high sympathetic tone to see if the pressor response of septic rats to this agent could be accentuated.

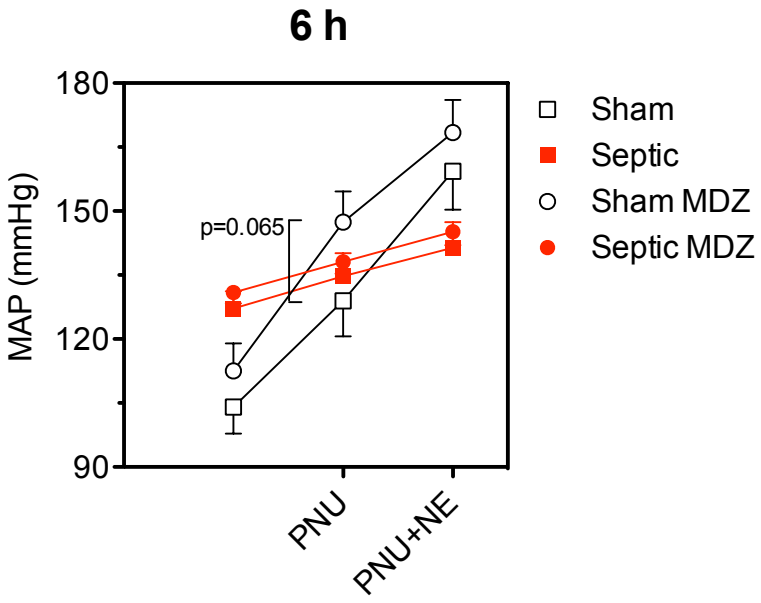


Figure 5.6. Mean arterial pressure (*MAP*) of 6h sham-operated and septic (faecal peritonitis) rats treated with PNU-37883A (*PNU*; 1.5 mg·kg^{-1} and 1.0 mg·kg^{-1}·h^{-1} i.v.) and norepinephrine (*NE*; 0.5 µg·kg^{-1}·min^{-1} i.v.), with or without an intravenous infusion of midazolam 0.2 mg·kg^{-1}·h^{-1} (*MDZ*). *Open and closed squares reproduce the same data as shown in* ***Figure 5.1c****.* n = *5 per group for rats receiving midazolam infusion.*

5.3.1. Pentolinium unmasked the PNU-37883A pressor response in septic rats

I used the autonomic ganglion blocker pentolinium to block sympathetic nerve output and thus investigated its effects in sham and septic rats at 6h (Figure 5.7). Infusion of pentolinium caused an immediate drop in blood pressure as shown by the example in Figure 5.7**a**. The septic rats had a larger drop in MAP than the sham animals, probably resulting from a significantly higher baseline blood pressure compared to shams (p <0.05) indicative of the presence of a higher sympathetic tone (Figure 5.7**b**). Furthermore, PNU-37883A elevated

blood pressure in pentolinium-infused sham rats to the same level as that measured in sham rats before the addition of pentolinium (Figure 5.7**b**).

The pressor response to PNU-37883A in the pentolinium-infused septic rats was, however, accentuated compared to that seen in animals not pre-treated with pentolinium (Figure 5.7), even though it was still smaller compared to sham rats (Figure 5.7**b**). When infused with the same dose of NE, both sham and septic rats pre-treated with pentolinium had an increase in MAP that was much larger than without pre-treatment, indicating successful sympathetic inhibition. Thus, sepsis-induced vasorelaxation and vasoplegia may have had less to do with increased vascular K_{ATP} channel because of its inhibition by sympathetic activation and could therefore explain why PNU-37883A showed no pressor effect in septic animals in this model.

To clarify that the potentiated pressor response to PNU-37883A in septic rats was not simply related to a lower baseline blood pressure, I re-tested it in the presence of two vasodilators that are not thought to directly affect sympathetic tone, namely sodium nitroprusside (SNP) and hydralazine. Under these conditions, PNU-37883A produced a similar pressor response, thus confirming that the potentiated pressor response to PNU-37883A seen with pentolinium could not be achieved by simply lowering blood pressure to a similar degree with either SNP or hydralazine (Figure 5.7**c**). Of note, this manoeuvre did reduce the pressor response of PNU-37883A in sham rats. The reason for this was unclear but might reflect a reflex stimulation of the sympathetic tone in

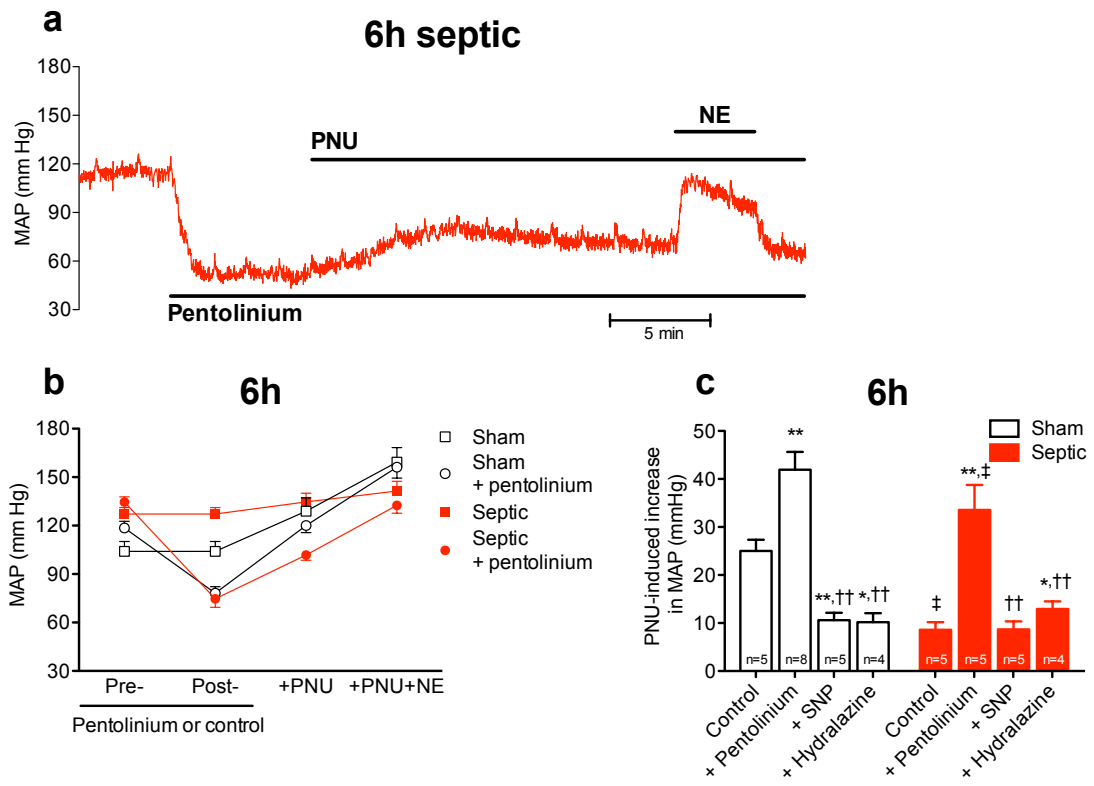


Figure 5.7. a) A representative blood pressure (*MAP*) trace of a septic rat with faecal peritonitis before and after administration of pentolinium (2 mg·kg^{-1} followed by mg·kg^{-1}·h^{-1} i.v.), PNU-37883A (PNU; 1.5 mg·kg^{-1} and 1.0 mg·kg^{-1}·h^{-1} i.v.), and norepinephrine (NE; 0.5 µg·kg^{-1}·min^{-1} i.v.), at the 6h timepoint; b) Effect of PNU-37883A ± NE on MAP in sham and septic rats at 6h, either pre-treated or not with pentolinium; c) changes in MAP induced by PNU-37883A in sham and septic rats at 6h, with or without pre-treatment with pentolinium, sodium nitroprusside (SNP; 50 µg·kg^{-1}·min^{-1} i.v.), or hydralazine (300 µg·kg^{-1}·min^{-1} i.v.). *p <0.05, **p <0.01 compared to baseline pressure; ††p <0.01 compared to the changes in pressure after pentolinium administration; ‡p <0.05 compared to sham animals at the same timepoint (two-way ANOVA repeated measures with Bonferroni post-hoc test).*

these animals (Figure 5.7**c**). In the septic rats pentolinium was also able to augment the pressor response to SEITU (Figure 5.8**a**), and to improve subsequent responsiveness to PNU-37883A (Figure 5.8**b**). This implies that vascular K_{ATP} channel responsiveness may be restored following removal of sympathetic tone and NOS activity. Surprisingly in sham rats PNU-37883A had no pressor response in the presence of SEITU.

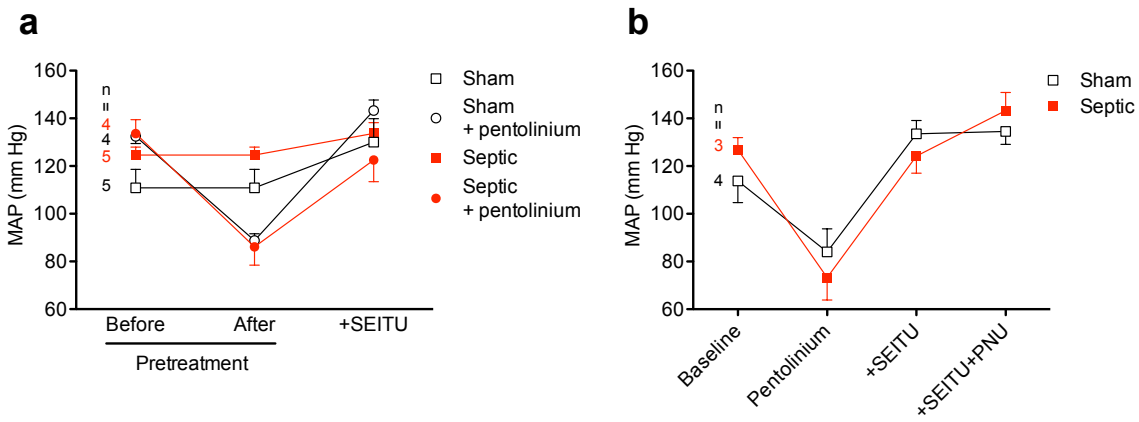


Figure 5.8. a) Effect of* S-ethylisothiourea *(*SEITU*; 0.1 mg·kg^{-1}·min^{-1}* i.v.*) ± norepinephrine (*NE*; 0.5 µg·kg^{-1}·min^{-1}* i.v.*) on mean arterial pressure (*MAP*) in sham and septic rats at 6h, either pre-treated or not with pentolinium; b) Effect of SEITU ± PNU-37883A (*PNU*; 1.5 mg·kg^{-1} and 1.0 mg·kg^{-1}·h^{-1}* i.v.*) on MAP in pentolinium-infused sham and septic rats at 6h.

5.3.2. Reserpinized rats

To confirm the above findings that the vascular K_{ATP} channel is inhibited by the high sympathetic tone in the awake septic rats, I tested the effects of PNU-

37883A in rats that had undergone chemical sympathectomy by subcutaneous injection of reserpine (Martínez-Olivares *et al.*, 2006).

A total of 22 rats (mean weight, 306 ± 5 g) were pre-treated with reserpine. Of these animals, eight were used as sham-operated controls, and 14 had peritonitis induced in them. Prior to induction of sepsis, these animals showed marked weight loss (∆56 ± 2 g; equivalent to 18.3% body weight loss) within 48h. All the reserpinized animals were set up for 6h experiments. Sham-operated rats presented with metabolic alkalosis (arterial base excess 4.1 ± 0.9 mM, n = 6, p <0.05 vs. 6h non-reserpinized rats) and an elevated plasma lactate level (2.9 ± 0.3 mM, n = 5, p <0.05), whereas septic rats presented with a more severe metabolic acidosis (arterial base excess -8.6 ± 1.3 mM, n = 6, p <0.01) and higher *in vivo* NO production (plasma NOx 78.4 ± 13.9 µM, n = 5, p <0.01). Septic rats had a 6h mortality rate of 28.6% (p <0.01), whereas none of the sham died at this timepoint.

All rats presented with a lower blood pressure at the time of faecal slurry injection (101.6 ± 2.7 mmHg for sham rats, n = 8, p <0.05 vs. non-reserpinized animals; 102.3 ± 4.2 mmHg for septic rats, n = 11, p <0.01). At the 6h timepoint, sham rats responded to fluid resuscitation and their blood pressure was elevated (112.8 ± 3.6 mmHg, p <0.01 vs. 0h), whereas septic rats remained hypotensive (105.2 ± 5.6 mmHg).

I intended to demonstrate that septic reserpinized rats had an accentuated pressor response to PNU-37883A, which could support the notion that the high

sympathetic tone presented in sepsis inhibits vascular K_{ATP} channel activity *in vivo*. Unfortunately the results were not as predicted (Figure 5.9). This may be due to the unexpected effect of reserpine that rendered the animals 'dehydrated,' evident indirectly by the marked weight loss and the presence of metabolic alkalosis in sham animals (Lecompte *et al.*, 1978). Despite this, sham rats still had a potentiated pressor response to PNU-37883A, albeit not statistically significant (Figure 5.9**b**). Reserpine did improve responses to NE in septic rats (Figure 5.9**a**). This may be related to upregulation of adrenergic receptors or a reduction in desensitization induced by NE deprivation.

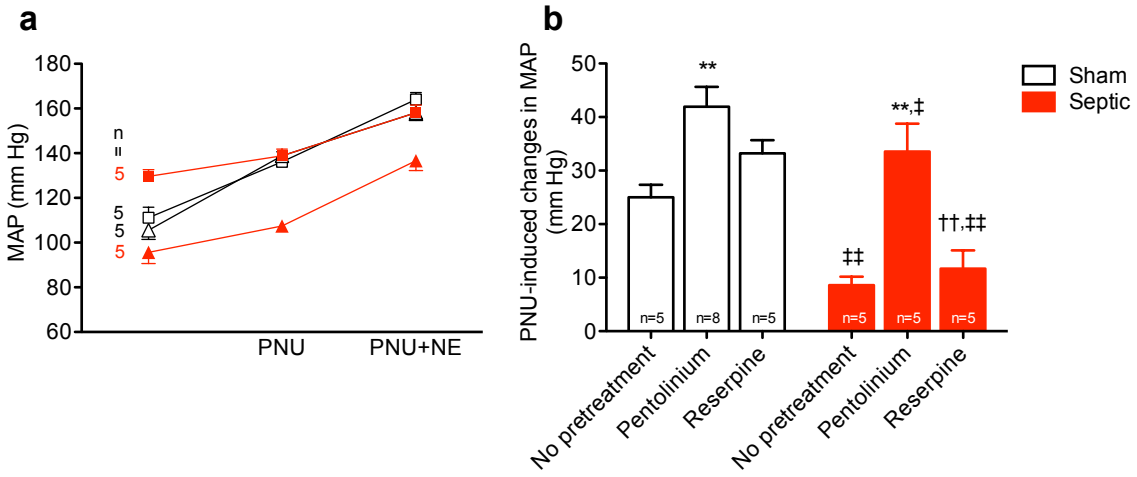


Figure 5.9. a) Changes in mean arterial pressure (MAP) in non-reserpinized (squares) and reserpinized (triangles) sham and septic rats induced by PNU-37883A (PNU; 1.5 mg·kg^{-1} and 1.0 mg·kg^{-1}·h^{-1} i.v.), with and without norepinephrine (NE; 0.5 µg·kg^{-1}·min^{-1} i.v.). b) Changes in MAP induced by PNU-37883A in sham and septic rats that received no pre-treatment, pre-treated with pentolinium, and pre-treated with reserpine. **p *<0.01 compared to appropriate no pre-treatment groups;* ††p *<0.01 compared to septic rats pre-treated with pentolinium;* ‡p *<0.05,* ‡‡p *<0.01 compared to sham rats under the same condition (two-way ANOVA repeated measures with Bonferroni* post-hoc *test).*

5.4. Discussion

Perhaps the most striking finding in this particular study was that the autonomic ganglion blocker, pentolinium, substantially enhanced the pressor response to PNU-37883A in the septic rats. This suggests that the high sympathetic tone presenting in sepsis can inhibit vascular K_{ATP} channel activity *in vivo*. This inhibition may originate from PKC blockade of K_{ATP} channel activity (Bonev and Nelson, 1996; Hayabuchi *et al.*, 2001; Thorneloe *et al.*, 2002) since vaso-constrictor receptors coupled to G_q will increase PKC activity through the second messenger diacylglycerol. Increased calcineurin activity (Wilson *et al.*, 2000; Orie *et al.*, 2009) resulting from an elevated $[Ca^{2+}]_i$ will also inhibit the K_{ATP} channel. Consistent with this notion, intracellular Ca^{2+} has been reported to rise in arterial smooth muscle during sepsis (Song *et al.*, 1993) and the host lab have also observed this in the faecal peritonitis model (Barrett *et al.*, unpublished observations).

Little attention has been drawn to changes in PKC activity during sepsis, perhaps because vascular K_{ATP} channel activity has always been deemed to be increased and the focus has, to date, fallen heavily on this aspect. Indeed, animals, *ex vivo* tissues or cultured primary cells made septic often present with accentuated responses to KCOs (Sorrentino *et al.*, 1999; Chen *et al.*, 2000; Wilson *et al.*, 2000; Shi *et al.*, 2010). However, only an increase in basal activity, not the stimulated activity, can cause the vascular changes. Although smooth muscle hyperpolarization has been observed in *ex vivo* arteries from septic rats (Chen *et al.*, 2000; Farias *et al.*, 2002; Wu *et al.*, 2004; Kuo *et al.*,

2009), importantly, these findings were made in tissues from which sympathetic neural input had been removed.

In a similar manner, the vasodilatory nature of sepsis has been a main focus of attention. Many studies have examined the influence of excessive NO production, the degree of attenuation in the pressor response to NE and, in clinical investigations, the reduced level of systemic vascular resistance. However, such subjects are still likely to have a high sympathetic tone, and their vascular K_{ATP} channel may become inhibited. Intravenous infusion of dopamine or NE as a therapeutic effort to correct septic shock may pose further inhibitory effects. This may possibly explain the failure of vascular K_{ATP} channel blockers in human sepsis (Warrillow *et al.*, 2006; Morelli *et al.*, 2007).

While septic rats seemed to have an accentuated hypotensive response to Lev at 6h, this effect curiously diminished at 24h. This is in directly contrast to the $^{86}Rb^{+}$ efflux experiment, which demonstrated an augmented response to Lev in arteries from 24h septic rats (Figure 3.7). This discrepancy may be attributed to the lower blood pressure in the 24h septic rats that prevented them from becoming further hypotensive, or other compensatory mechanisms intervened and rendered Lev less effective in opening the channel. For example, calcineurin may directly oppose the effect of PKA (Orie *et al.*, 2009) and thereby prevent the response to Lev from being potentiated (Kessler *et al.*, 1997). Anyhow, the major implication is that the *in vivo* activity of the vascular K_{ATP} channel is not in parallel with that measured *in vitro*, and may change with time and disease severity.

Another important finding in this present work was that the pressor effect of PNU-37883A in sham rats was dependent on NOS activity. When co-administered with SEITU, the pressor effect of PNU-37883A was attenuated in non-reserpinized sham rats, and in reserpinized sham animals, it was even abolished by SEITU. NO derived from NOS3 may activate endothelial (Wang *et al.*, 2007) and/or smooth muscle K_{ATP} channels. Septic rats, reserpinized or not, still showed some pressor responses to PNU-37883A. This suggests that the vascular K_{ATP} channel activity in septic rats was not solely dependent on NOS activity; other stimuli present in sepsis could also open the channel. NOS3 activity was the NOS activity possibly inhibited by SEITU in sham rats.

In sham rats pre-treated with SNP and hydralazine, the pressor effect of PNU-37883A was attenuated. This could be attributed to a reflex increase in sympathetic tone that subsequent inhibited the channel. However, it is also conceivable that arteries in these animals were simply relaxed too profoundly to become responsive to PNU-37883A. To further clarify the notion that high sympathetic tone inhibits vascular K_{ATP} channel hyperactivity *in vivo*, I should either stimulate the sympathetic nervous system of the sham rats and demonstrate an attenuated the pressor response to PNU-37883A, or to remove the high sympathetic tone in the septic animals to restore the pressor effect of this agent. Therefore I tested the effect of PNU-37883A in rats receiving reserpine as a means of chemical sympathectomy. I did not use the α-adrenergic receptor antagonist phentolamine as this inhibits the K_{ATP} channel (Russell *et al.*, 1992; Ibbotson *et al.*, 1993; Proks and Ashcroft, 1997). I did not either use the other α-adrenergic receptor antagonists (*e.g.* prazosin, phenoxy-

benzamine) because my intention was to deplete their NE reserves from the sympathetic nerves, so that the difference in levels of sympathetic inhibition would be minimized, and a lack of accentuation in the pressor response to PNU-37883A would not be originated from an insufficient sympathetic blockade. I did not use the muscarinic cholinergic receptor antagonist (*e.g.* atropine) because most blood vessels do not have parasympathetic innervation (Guyton and Hall, 2006). That reserpinized rats seemed to develop volume depletion was totally unexpected, which might cause the under-estimation of the pressor effect of PNU-37883A and render me unable to prove my point.

In summary, the activity of the vascular K_{ATP} channel may be inhibited by the high sympathetic tone present in sepsis. I hypothesise this would occur through induction of PKC and calcineurin activity, although time precluded me from directly testing this. This inhibition can be removed by the autonomic ganglion blocker, pentolinium that can unmask the pressor effect of PNU-37883A in septic rats. Although the same changes could not be reproduced in reserpinized septic rats, this may be possibly due to their greater degree of volume depletion; indeed, reserpinized sham rats had a larger pressor response to PNU-37883A. In sham rats, the pressor effect of PNU-37883A appears to depend on NOS activity. Evidence is lacking to support the involvement of BK_{Ca} channel activity in sepsis-induced vascular changes in this model.

Chapter Six Summary and Discussion

6.1. Summary of results

The initial aim of the current project was to demonstrate that sepsis would increase vascular K_{ATP} channel gene expression and this would contribute to channel hyperactivity and vasorelaxation. While rendering animals septic to demonstrate the presence of channel induction *in vivo*, I also sought to confirm the therapeutic efficacy of vascular K_{ATP} channel pore blockade to reverse sepsis-induced vasoplegia. Unexpectedly, septic rats had a profoundly attenuated pressor response to PNU-37883A, and this attenuation could be partially reversed by the autonomic ganglion blocker, pentolinium. Reserpinized sham rats, although markedly dehydrated, still seemed to have an accentuated pressor response to PNU-37883A, suggesting that the resting sympathetic tone can pose some inhibitory effect on the vascular K_{ATP} channel. Since septic patients and animals often have a high sympathetic tone, it may well be that during the course of disease their vascular K_{ATP} channels remain inhibited owing to the consequence of increased PKC activity, plus an increased calcineurin activity resulting from an elevated $[Ca^{2+}]_i$ in arterial smooth muscle. In this respect, the vascular K_{ATP} channel may have less contribution to the sepsis-induced vascular changes. However, I cannot rule out the possibility that sepsis reduces the ability of agents like PNU-37883A to block the channel which may mask or under-estimate K_{ATP} channel involvement.

Regarding vascular K_{ATP} channel induction, I found a fourfold increase in $K_{IR}6.1$ subunit gene expression in mesenteric arteries from 24h septic rats, whereas in aortae the gene expression of $K_{IR}6.1$ appeared unchanged. For SUR2B, there was a decrease in gene expression in aortae from septic rats at 6h, otherwise it remained unchanged throughout. The increase in $K_{IR}6.1$ transcriptional expression may not correlate well with the concurrent change in K_{ATP} channel activity, as the number of the newly generated channels may be limited by the amount of SUR2B reserve in the arterial smooth muscle cell. Besides, aortae from 24h septic rats had an increase in levcromakalim-stimulated K_{ATP} channel activity, but no increase in channel gene expression, suggesting that other mechanisms such as alteration in channel kinetics might be involved. Even if the number of channels in the plasma membrane is indeed increased owing to sepsis-related gene expression, the channels are likely to be inhibited by the high sympathetic tone present in sepsis, thus posing a smaller effect on the vasculature. One concern might be when agents able to open the vascular K_{ATP} channel (*e.g.*, isoflurane, KCOs, and cyclosporin) are administered to septic patients or animals, this may accentuated the hypotensive effect, thus potentially worsening their condition. Nevertheless, pinacidil, another K_{ATP} channel opener, appeared to improve the mortality of endotoxaemic mice (Kane *et al.*, 2006).

An additional finding is that the pressor effect of PNU-37883A was much attenuated in sham rats treated with SEITU, suggesting that physiological activity of the vascular K_{ATP} channel is dependent on NOS3 activity. This suggests that the activity of the channel *in vivo* may be higher than that

observed in *in vitro* preparations. Also in both the absence and presence of pentolinium, SEITU had a more profound pressor effect than PNU-37883A in both sham and septic rats. This suggests that NO may contribute to the regulation of vascular tone through multiple pathways in addition to vascular K_{ATP} channel stimulation.

6.2. Discussion

The vascular K_{ATP} channel can be activated by various vasodilating stimuli, many of which become elevated during sepsis. Since channel hyperactivity can lead to vasorelaxation that is pathognomonic of septic shock, it seems a reasonable conjecture that during sepsis the channel is activated. However, this notion is directly controverted by the experimental finding of an elevated $[Ca^{2+}]_i$ in aortic smooth muscle from septic rats (Song *et al.*, 1993; Barrett *et al.*, unpublished observations), which would have the effect of inhibiting channel activity (Orie *et al.*, 2009). This begs the question whether vascular K_{ATP} channel hyperactivity and the consequent inhibition of voltage-dependent Ca^{2+} channel (VDCC) can exist at the same time with an elevated $[Ca^{2+}]_i$ in arterial smooth muscle? Moreover, does arterial smooth muscle actually present with membrane hyperpolarization and VDCC inhibition in sepsis?

As discussed previously, the membrane hyperpolarization of vascular smooth muscle in response to septic stimuli (Chen *et al.*, 2000; Farias *et al.*, 2002; Wu *et al.*, 2004; Kuo *et al.*, 2009) was demonstrated in *ex vivo* tissues, to which the counteracting/depolarizing effect of sympathetic tone has been removed.

Whether the measured E_m could reflect its true level *in vivo* remains questionable. Regarding the activity of VDCC during sepsis, (Chen *et al.*, 2010) found that aortae collected from caecal ligation and puncture (CLP) rats showed augmented response to the VDCC inhibitor nifedipine, which was reversible by L-NAME. This suggested NO activates VDCC in these animals, a result in contrast with the general idea that NO donors inhibit VDCC (Clapp and Gurney, 1991; Carvajal *et al.*, 2000). In contrast, peak L-type Ca^{2+} current density was reduced in cardiac myocytes isolated from a porcine model of hyperdynamic septic shock (Stengl *et al.*, 2010). Thus whether the activity of VDCC in sepsis is suppressed or activated remains to be clarified.

Whether the vascular K_{ATP} channel is inhibited *in vivo* by the high sympathetic tone? Sham rats rendered hypotensive with sodium nitroprusside and hydralazine showed an attenuated pressor response to PNU-37883A; in contrast, reserpinized sham rats showed an accentuated response as the inhibitory effect from the sympathetic output was removed. These findings almost represent a continual levels of inhibition that dependents on sympathetic tone. The pressor response of septic rats to PNU-37883A could also be partially unmasked by pentolinium, which induced a greater drop in MAP in these animals suggesting the presence of a higher sympathetic tone. This is the first time that the activity of vascular K_{ATP} channels has been reported to be related to the sympathetic tone *in vivo*. It also appears that the vascular K_{ATP} channel, stimulated by physiological activity of NOS3, has a basal activity higher than previously suggested. Thus the channel may assume a more important and active role in the physiological regulation of vascular tone.

High sympathetic tone may also exert other effects to attenuate pressure elevation from vascular K_{ATP} channel blockade. The background arterial smooth muscle E_m should be more depolarized in septic rats with a higher sympathetic tone. Inhibition of the vascular K_{ATP} channel may further depolarize the muscle, but the resultant increase in P_o of VDCC might be less prominent, as at this level of E_m the increase in P_o tends to be offset by channel inactivation and decrease in driving force (Nelson *et al.*, 1990). Also, NE can directly stimulate VDCC (Nelson *et al.*, 1990). This and the activation of VDCC induced by membrane depolarization, together with a functional impairment of sarcoplasmic reticulum Ca^{2+}-ATPase (Walia *et al.*, 2003), contribute to elevated $[Ca^{2+}]_i$ in arterial smooth muscle. Even so, the muscle remains relaxed, suggesting presence of Ca^{2+} desensitization which renders elevated $[Ca^{2+}]_i$ less capable of increasing contractility, but a decrease in $[Ca^{2+}]_i$ more capable of relaxing the muscle. Thus in septic rats, a higher $[Ca^{2+}]_i$ and Ca^{2+} desensitization may render vascular K_{ATP} channel blockade less effective in mediating vasoconstriction.

In this regard, it seems reasonable that SEITU can exert a more potentiated pressor effect than PNU-37883A in septic rats and as SEITU would decrease cGMP and is thereby able to reverse Ca^{2+} desensitization. In addition, SEITU can also contract the venous vasculature which augments cardiac preload, whereas PNU-37883A probably cannot because venous smooth muscle does not appear to express K_{ATP} channels (Li *et al.*, 2003). However, an increase in cardiac preload should bring about an increased cardiac output, but SEITU was actually found to decrease cardiac output (Dyson A, unpublished observations)

like that observed with other NO inhibitors (Vincent *et al.*, 2000). Thus it remains uncertain whether NOS blockade can actually augment cardiac preload.

Indeed, the revelation of effects of sympathetic tone on vascular K_{ATP} channel activity is indebted to the high sympathetic tone of septic rats at 6h, which was accompanied by an elevated blood pressure. Even though septic rats developed hypotension at 24h, the sympathetic tone is likely to be high as these animals still were tachycardic. Whether rats have a higher sympathetic tone compared to humans, and whether this can explain the stronger inhibitory effect on vascular K_{ATP} channel, remains to be determined. Plasma concentration of NE is frequently used as a marker of sympathetic activity. NE in plasma represents that secreted from adrenal medullae and that spilled into circulation from the junctions between postsynaptic neurons and end effector organs. The spillover reflects not only NE release from nerve terminals but also reuptake processes (Kopin *et al.*, 1998). Indeed, much of the overall sympathetic tone originates from basal secretion of epinephrine and NE from adrenal medullae, with quantities sufficient to maintain the blood pressure up to normal even if the direct sympathetic stimulation to the cardiovascular system is removed (Guyton and Hall, 2006). NE represents 20% of catecholamines secreted from adrenal medulla, and the normal resting rate of secretion is about 0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (Guyton and Hall, 2006). The normal plasma concentration of NE in humans is around 300-400 $pg \cdot ml^{-1}$ (Dimsdale and Moss, 1980), which is similar to that of rats (Barrett *et al.*, 2007a). Rats subject to faecal peritonitis had a plasma NE level of about 2,000 $pg \cdot ml^{-1}$ beyond 24h, but the level was only about 600 $pg \cdot ml^{-1}$ at 4h, when septic rats already had elevated blood pressure (Barrett *et al.*,

2007a). Patients with septic shock or severe sepsis had even higher levels of NE when presented to the emergency department (about 3,600 $pg \cdot ml^{-1}$ for severely septic patients, Lin *et al.*, 2005). On this basis, severely septic patients also have a high sympathetic tone, and possibly even higher than rats. Not to mention the therapeutic use of NE and dopamine as a key component of clinical resuscitation and treatment of septic shock. The therapeutic doses of NE ranges from 0.05 to 1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ *i.v.*, but can escalate to 3 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (Hollenberg *et al.*, 2004). Infusion of 2.5 nmol (0.4 $\mu g) \cdot kg^{-1} \cdot min^{-1}$ of NE increased the plasma NE level to about 11 $ng \cdot ml^{-1}$ (Hjemdahl *et al.*, 1979). The plasma NE level of non-surviving patients sustaining severe trauma or postoperative complications could become as high as 10 $ng \cdot ml^{-1}$ with administration of exogenous catecholamines (Boldt *et al.*, 1995). With such high levels of sympathetic stimulation, the vascular K_{ATP} channel will be profoundly inhibited, making the channel less likely to contribute to vasorelaxation.

Also the effect of pentolinium to accentuate the pressor response of septic rats to PNU-37883A may result from its blockade of parasympathetic output, which may also be high in sepsis (Shoemaker *et al.*, 2005). Parasympathetic tone, through the release of agents like acetylcholine could inhibit the vascular K_{ATP} channel through PKC activity (Bonev and Nelson, 1993b; Quinn *et al.*, 2004). However, in the absence of direct innervation, how much acetylcholine could be delivered to the vascular smooth muscle and exert the K_{ATP} channel-inhibitory effect remains uncertain, meanwhile the NO generated by the endothelium as stimulated by acetylcholine may offset this inhibition. How parasympathetic

nerve activity modulates vascular K_{ATP} channels and vascular behaviour during sepsis remains to be investigated.

Figure 6.1 shows a schematic representation of interactions between vasoactive stimuli, $[Ca^{2+}]_i$ and vascular K_{ATP} channel activity during sepsis. Sepsis can stimulate both vasoconstricting and vasodilating mechanisms simultaneously. Each mechanism imposes their respective effects on the channel, and on intracellular Ca^{2+} mobilization and homeostasis. Calcineurin activity can be stimulated by high $[Ca^{2+}]_i$, which can impose an additional inhibition upon vascular K_{ATP} channel activity.

Nevertheless, results from Kane *et al.* (2006) seem to point out why $K_{IR}6.1$ subunit gene would be induced during sepsis. To simply put, without this increase in gene expression, septic animals may have died of coronary spasm. In other words, this increase in $K_{IR}6.1$ gene expression may serve as a protective mechanism against overt vasoconstriction induced by sympathetic hyperactivity. Again, it seems odd to utilize gene expression as a compensatory mechanism to functional derangement. Nevertheless, mice carrying null alleles of $K_{IR}6.1$ became more susceptible to bacterial endotoxin in causing death, which seemed again attributable to an increased coronary vasoconstriction. Moreover, fruit flies being suppressed of the *Abcc9* (SUR2) homolog by RNA-mediated interference became more susceptible to flock house virus infection in lethality, again attributable to an intrinsic heart defect (Croker *et al.*, 2007). Therefore, surviving from sepsis seems indeed dependent on the presence of functioning vascular K_{ATP} channels.

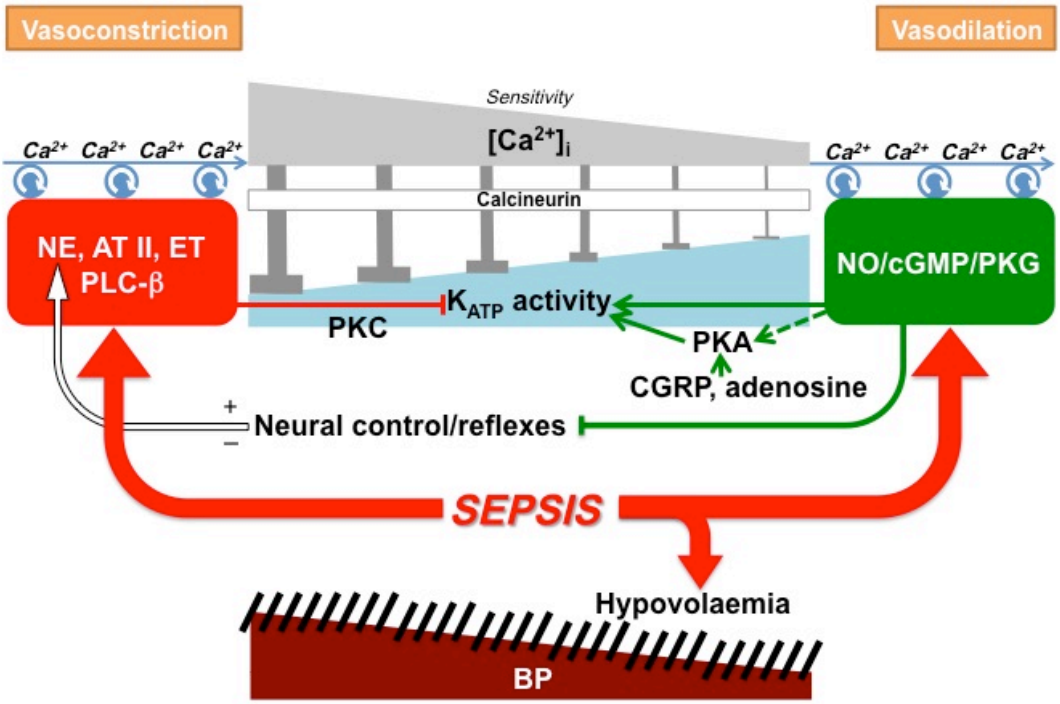


Figure 6.1. Interactions between vasoactive stimuli, $[Ca^{2+}]_i$ and vascular K_{ATP} channel activity during sepsis. *Vasoconstricting stimuli (e.g., norepinephrine [*NE*], angiotensin [*AT*] II and endothelin [*ET*]) increase $[Ca^{2+}]_i$ through phospholipase C (*PLC*)-β activity. These stimuli also inhibit vascular K_{ATP} channels through protein kinase C (*PKC*) activity. Vasodilating stimuli (e.g., nitric oxide [*NO*], calcitonin gene-related peptide [*CGRP*] and adenosine) decrease $[Ca^{2+}]_i$ through cAMP-dependent protein kinase* (PKA*) and cGMP-dependent protein kinase (*PKG*) activity; they can also stimulate vascular K_{ATP} channels through the activities of these enzymes, or directly through NO which can also modulate autonomic neural control and reflexes. An elevated $[Ca^{2+}]_i$ also activates calcineurin activity, which is inhibitory to vascular K_{ATP} channels. It is thus likely that the K_{ATP} channel activity in vivo is mostly inhibited rather than activated in sepsis, even though both vasoconstricting and vasodilating mechanisms simultaneously impose their respective effects on the channel. Administering channel inhibitors can be potentially dangerous, as any remaining K_{ATP} channel activity may help to prevent the occurrence of coronary vasospasm. Hypovolaemia will also affect blood pressure, and could also confound results if blood pressure is used as a marker of sepsis-induced vascular changes.*

The small sample sizes of many experiments in this study can constitute two serious problems, one is the low statistic power of the test to differentiate the true difference, and the other the under-estimation of biological variability, *i.e.* a small standard deviation of a measurement in a small-sized sample raises doubts as to whether this sample is representative enough to demonstrate the variability of the measurement. Most of my results showed significant differences without being affected by the low statistic power, probably except for the basal $^{86}Rb^{+}$ efflux at 24h (discussed in **3.5**), the increase in blood pressure in sham rats receiving midazolam and PNU-37883A, and that in reserpinized sham rats receiving PNU-37883A. Even though the main results remain the same, this concern should have been avoided during the setup of the experiment. The biological variability of the model was actually greater than I predicted, despite these rats were rather homeogenous: young, healthy, and bred in a stable environment. For example, the standard deviation of blood pressure in 6h sham rats was 15 mmHg (n = 47). Thus a larger sample size can cover a wider range of biological variability and allow more accurate estimation of the population.

The major limitation of this project is that the model and the means of measurements used were subsequently found to be substantially confounded. Rats in the faecal peritonitis model may have been confounded by intravascular volume depletion, and changes in blood pressure seems not the most appropriate means to measure sepsis-induced vasorelaxation/vasoplegia. $^{86}Rb^{+}$ efflux experiments were performed on *ex vivo* arterial tissues devoid of sympathetic stimulation and NOS activity, and the experiment itself was not

sensitive enough to detect changes in basal vascular K_{ATP} channel activity. Reserpinized rats unexpectedly developed severe hypovolaemia that confounded the blood pressure and vascular reactivity to vasopressors, and Western blotting could not accurately reflect changes in channel numbers in the plasma membrane. I have no evidence that directly supports the notion that vascular K_{ATP} channel is inhibited by the high sympathetic tone present in septic animals, and I am not certain that if there exists arterial smooth muscle hyperpolarization exists in septic animals and is sensitive to PNU-37883A.

6.3. Future investigations

An important lesson learned from this project is that the exact physiological events that occur *in vivo* cannot be accurately and completely reproduced *in vitro*. This could potentially lead to misinterpretation of experimental results and misconception of disease processes. It is still not entirely clear whether arterial smooth muscle actually develops membrane depolarization or hyperpolarization during sepsis *in vivo*, whether vascular K_{ATP} channels are activated by NO or suppressed by PKC and calcineurin activity *in vivo*, and to which level and through which mechanisms can sympathetic nervous system be activated by sepsis *in vivo*. Moreover, all these conditions could be affected by disease, pathogen, host and temporal factors, which should also be addressed. (Chen and Rivers, 2001) already devised a method that allows measurement of arteriolar smooth muscle membrane potential and $[Ca^{2+}]_i$ *in vivo*. I would love to see if septic animals actually develop glibenclamide- or PNU-37883A-sensitive hyperpolarization during early and prolonged sepsis, and if sympathetic

inhibition in septic rats or sympathetic stimulation in sham rats would alter these membrane potential changes and corresponding $[Ca^{2+}]_i$. Besides, I would also like to test some means that are capable of modulating PKC and calcineurin activity *in vivo,* so as to confirm the cause-effect relationship between the high sympathetic tone present in sepsis and the inhibition of vascular K_{ATP} channel activity.

Nevertheless, what seems more important to me as a clinician is to find out what causes septic shock, and how can it be reversed. The use of catecholamines to reverse hypotension cannot be fully justified (Singer, 2007), whereas NOS blockade (Lopez *et al.*, 2004) and vasopressin administration (Russell *et al.*, 2008) have not yet been proven beneficial over and above what catecholamines can do. Septic shock features arterial and venous vasodilatation, but baroreflex intact dogs were capable of compensating the haemodynamic instability resulting from the diffuse arteriolar dilatation induced by dinitrophenol (Banet and Guyton, 1971). Is there a defect in baroreflex that deranges this compensation during sepsis? Moreover, decapitated and spinal cord-destroyed dogs could increase their cardiac output as much as baroreflex intact animals if their blood pressure could be maintained through intra-arterial transfusion, suggesting sufficient venous return is extremely important in maintaining cardiac output provided myocardial contractility remains normal. Sepsis can induce venodilatation and vascular leak that decrease venous return, and also cardiac dysfunction that decreases the heart's ability to compensate for hypotension. Importantly, intravenous fluid resuscitation in this setting may become less efficient if venodilatation and consequent increase in

vascular compliance are not controlled. It seems to me that these factors are important in causing/contributing to septic shock but are seldom addressed. I would like to further explore the change in venous vasculature and baroreflex that occurs during sepsis, its underlying mechanism, and the consequent alterations in haemodynamics and impact in disease outcomes.

6.4. Concluding remarks

The results of the study have highlighted the complexity of pathophysiology of septic shock, and the profound effect of sympathetic hyperactivity that poses on vascular smooth muscle. Intracellular Ca^{2+} handling in arterial smooth muscle can be affected by several mechanisms, and with disease progression and incremental metabolic burdens, derangements in individual mechanisms can vary in degree, rendering efficacy of rescue measures variable and inconsistent. *In vivo* models may not be suitable for dissecting out the effect of individual mechanisms; however, findings from *in vitro* studies may always need to be validated in an *in vivo* model that essentially reproduce clinical disease state and pathophysiology. The complexity of pathophysiology of septic shock should not be underestimated, and further studies are required to first define which characteristics amongst septic shock patients are more consistently present and significant that need to be reproduced in animal models. Equally important is the nature of the animal model itself: would any particular behaviour of the model confound the result?

The exploration of vascular K_{ATP} channel during sepsis was based on the thinking that the channel is an important regulator of vascular tone, and the channel can be inhibited by glibenclamide, an oral hypoglycaemic agent that has long been used clinically to treat type II diabetes mellitus. Although the rationale that the channel activated by NO facilitates vasodilatation during sepsis seems absolutely sensible, an equally important reason may be the readily availability of the therapeutic agent. Indeed, glibenclamide was used clinically to reverse haemodynamic instability induced by agents that pharmacologically open the channel (Singer *et al.*, 2005), a result that was extremely persuasive and encouraging. It is apparent now that during sepsis the vascular K_{ATP} channel may or may not be activated, and glibenclamide no longer the 'magic bullet' that is able to attract people's attention. Perhaps it would be prudent to look back to the molecular events of intracellular Ca^{2+} handling in more detail during sepsis, and to first determine the relationship between VDCC activity and arterial smooth muscle E_m in the presence of high sympathetic tone and cGMP/PKG activity, before focusing on vascular K_{ATP} channels as a potential therapeutic target for septic shock.

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